TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. § 371	Attorney's Docket Number 056222-5008
	U.S. Application No. Unassigned 10/048228

International Application. No.	International Filing Date	Priority Date Claimed
PCT/GB00/02946	July 31, 2000	July 29, 1999

Title of Invention: METHOD FOR AMPLIFICATION OF NUCLEIC ACIDS

Applicants For EO/EO/US: John Scott LLOYD, Anthony WESTON, Donald Leonard Nicholas CARDY and Peter MARSH

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. § 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. § 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. § 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. § 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. § 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. § 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. § 371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 371(c)(3)).
9. ☐ An oath or declaration of the inventors (35 U.S.C. § 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. § 371(c)(5)).

Items 11. to 14. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C.F.R. § 1.97 and § 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. § 3.28 and § 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☒ Other items or information:
    a. PCT/IPEA/409
    b. PCT/IB/304
    c. PCT/IB/308
    d. Statement Accompanying Sequence Listing
    e. Diskette containing Sequence Listing CRF
    f. Paper Copy of Sequence Listing

U.S. APPLICATION NO.	INTERNATIONAL APPLICATION NO.	ATTORNEY DOCKET NUMBER
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15. ☒ The following fees are submitted:

Basic National Fee (37 C.F.R. § 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO or JPO.....$890.00
International preliminary examination fee paid to USPTO (37 C.F.R. § 1.482).....$710.00
No international preliminary examination fee paid to USPTO (37 C.F.R. § 1.482) but international search fee paid to USPTO (37 C.F.R. § 1.445(a)(2)).....$740.00
Neither international preliminary examination fee (37 C.F.R. § 1.482) nor international search fee (37 C.F.R. § 1.445(a)(2)) paid to USPTO.....$1,040.00
International preliminary examination fee paid to USPTO (37 C.F.R. § 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT = $890.00

Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(e)). $

Claims	Number Filed	Number Extra	Rate	
Total Claims	26 - 20 =	6	X $18.00	$ 108.00
Independent Claims	1 - 3 =	0	X $84.00	$
Multiple dependent claim(s) (if applicable)			+ $280.00	$
			TOTAL OF ABOVE CALCULATIONS	$ 998.00
			Reduction by ½ for filing by small entity, if applicable.	**-$499.00**
			SUBTOTAL =	**$ 499.00**
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(f)).				+$
			TOTAL NATIONAL FEE =	**$499.00**
Fee for recording the enclosed assignment (37 C.F.R. § 1.21(h)). The Assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§ 3.28, 3.31). $40.00 per property				$
			TOTAL FEES ENCLOSED =	**$**
			Amount to be refunded	$
			Amount to be charged	**$ 499.00**

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 50-0310 in the amount of **$499.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ **Except** for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. § 1.16 and § 1.17 which may be required, or credit any overpayment to Deposit Account No. 50-0310.

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Submitted: January 29, 2002

PATENT
Attorney Docket No. 056222-5008

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of : John Scott **LLOYD**, et al.

U.S. National Phase Application
Filed: January 29, 2001

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Stage Entry : Concurrently

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For: METHOD FOR AMPLIFICATION OF NUCLEIC ACIDS

Group Art Unit: Unassigned

Examiner: Unassigned

Commissioner for Patents
Washington, D.C. 20231

Sir:

PRELIMINARY AMENDMENT

Prior to the examination of the above-identified application on the merits, please amend the application as follows:

IN THE CLAIMS:

Please substitute the following amended versions of claims 3-15, 18-21 and 23-26 for the original claims.

3. (AMENDED) A probe according to claim 1, comprising a –5 sequence adjacent to the 3' end of the promoter sequence.

4. (AMENDED) A probe according to claim 1, comprising a +12 sequence adjacent to the 5' end of the promoter.

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5. (AMENDED) A probe according to claim 1, such that when hybridised to the target, the 3' end of the target is extendible by a DNA polymerase.

6. (AMENDED) A probe according to claim 1, wherein the target complementary portion is located 3' of the promoter sequence.

7. (AMENDED) A probe according to claim 1, wherein a blocking moiety is located between position –19 and - 68.

8. (AMENDED) A probe according to claim 1, wherein a blocking moiety is located between position –19 and –38.

9. (AMENDED) A probe according to claim 1, wherein a blocking moiety is located between position –22 and –35.

10. (AMENDED) A probe according to claim 1, wherein the blocking moiety comprises a C_2-C_{20} alkyl, alkanol or alkylene residue.

11. (AMENDED) A probe according to claim 1, wherein the probe comprises a C_3-C_{10} alkyl, alkanol or alkylene residue.

12. (AMENDED) A probe according to claim 1, comprising an octanediol, propanediol or hexaethylene glycol residue.

13. (AMENDED) A probe according to claim 1, comprising PNA and/or LNA.

14. (AMENDED) A probe according to claim 1, wherein a target complementary

protein of the probe comprises PNA and/or LNA.

15. (AMENDED) A method of detecting a nucleic acid sequence of interest in a sample, the method comprising the steps of: contacting a nucleic acid probe molecule in accordance with claim 1 with a nucleic acid target molecule, which target is the sequence of interest or is formed as a result of the presence in the sample of the sequence of interest; causing extension of the 3' end of the target using the probe as a template, thereby creating a functional double stranded RNA polymerase promoter; causing RNA synthesis from the RNA polymerase promoter, to create an RNA molecule; and detecting directly or indirectly the RNA molecule so produced.

18. (AMENDED) A method according to claim 16, wherein the sequence of the further RNA molecule is substantially similar to that of the original target molecule, such that the further RNA molecule is able to hybridise, under the assay conditions employed, to the original nucleic acid probe molecule.

19. (AMENDED) A method according to claim 16, wherein the target sequence comprises DNA or RNA.

20. (AMENDED) A method according to claim 16, wherein the target sequence is DNA or RNA formed as a result of the presence in the sample of the nucleic acid sequence of interest.

21. (AMENDED) A method according to claim 16, wherein the RNA molecule is detected directly or indirectly by means of a labelled binding partner.

23. (AMENDED) A method according to claim 21, wherein the labelled binding

partner comprises DNA, RNA, LNA, PNA, or any combination thereof.

24. (AMENDED) A kit for use in performing a method of detecting a nucleic acid sequence of interest, comprising a probe molecule in accordance with claim 1, and packaging means.

25. (AMENDED) A kit according to claim 24, further comprising one or more of the following: instructions for performing the method; a buffer; a DNA polymerase; an RNA polymerase; deoxyribonucleotide triphosphates; ribonucleotide triphosphates; and a labelled binding partner.

26. A method of detecting a nucleic acid sequence of interest in a sample, the method comprising the steps of: contacting a nucleic acid probe molecule in accordance with claim 1 with a further probe and with a nucleic acid target molecule, which target is the sequence of interest or is formed as a result of the presence in the sample of the sequence of interest; causing the further probe molecule and the target molecule to hybridise adjacent each other to the probe molecule, thereby creating a functional double stranded RNA polymerase promoter; causing RNA synthesis from the RNA polymerase promoter, to create a RNA molecule; and detecting directly or indirectly the RNA molecule so produced.

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REMARKS

Applicants respectfully submit that no prohibited new matter has been introduced by this Preliminary Amendment and that amended claims 1 to 26 are drawn to the same invention as claims 1-26 of International Application PCT/GB00/02946. The changes to the claims represent changes in formalities so as to bring the claims into compliance with the rules of practice in the United States, avoiding improper multiple dependencies and eliminating multiple dependencies so as to reduce costs.

Respectfully Submitted,

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MARKED-UP VERSION TO SHOW CHANGES IN CLAIMS

3. **(AMENDED)** A probe according to claim 1 ~~[or 2]~~, comprising a –5 sequence adjacent to the 3' end of the promoter sequence.

4. **(AMENDED)** A probe according to ~~[any one of claims 1, 2 or 3,]~~ **claim 1,** comprising a +12 sequence adjacent to the 5' end of the promoter.

5. **(AMENDED)** A probe according to ~~[any one of the preceding claims]~~ **claim 1**, such that when hybridised to the target, the 3' end of the target is extendible by a DNA polymerase.

6. **(AMENDED)** A probe according to ~~[any one of the preceding claims]~~ **claim 1**, wherein the target complementary portion is located 3' of the promoter sequence.

7. **(AMENDED)** A probe according to ~~[any one of the preceding claims]~~ **claim 1**, wherein a blocking moiety is located between position –19 and - 68.

8. **(AMENDED)** A probe according to ~~[any one of the preceding claims]~~ **claim 1**, wherein a blocking moiety is located between position –19 and –38.

9. **(AMENDED)** A probe according to ~~[any one of the preceding claims]~~ **claim 1**, wherein a blocking moiety is located between position –22 and –35.

10. **(AMENDED)** A probe according to ~~[any one of the preceding claims]~~ **claim 1**, wherein the blocking moiety comprises a C_2-C_{20} alkyl, alkanol or alkylene residue.

11. **(AMENDED)** A probe according to ~~[any one of the preceding claims,]~~

11. **(AMENDED)** A probe according to ~~[any one of the preceding claims,]~~ **claim 1** wherein the probe comprises a C_3-C_{10} alkyl, alkanol or alkylene residue.

12. **(AMENDED)** A probe according to ~~[any oen of the preceding claims]~~ **claim 1** comprising an octanediol, propanediol or hexaethylene glycol residue.

13. **(AMENDED)** A probe according to ~~[any one of the preceding claims]~~ **claim 1**, comprising PNA and/or LNA.

14. **(AMENDED)** A probe according to ~~[any one of the preceding claims]~~ **claim 1**, wherein a target complementary protein of the probe comprises PNA and/or LNA~~[>]~~.

15. **(AMENDED)** A method of detecting a nucleic acid sequence of interest in a sample, the method comprising the steps of: contacting a nucleic acid probe molecule in accordance with ~~[any one of the preceding claims]~~ **claim 1** with a nucleic acid target molecule, which target is the sequence of interest or is formed as a result of the presence in the sample of the sequence of interest; causing extension of the 3' end of the target using the probe as a template, thereby creating a functional double stranded RNA polymerase promoter; causing RNA synthesis from the RNA polymerase promoter, to create an RNA molecule; and detecting directly or indirectly the RNA molecule so produced.

18. **(AMENDED)** A method according to claim 16 ~~[or 17]~~, wherein the sequence of the further RNA molecule is substantially similar to that of the original target molecule, such that the further RNA molecule is able to hybridise, under the assay conditions employed, to the original nucleic acid probe molecule.

19. **(AMENDED)** A method according to ~~[any one of claims]~~ **claim** 16, ~~[17 or 18,]~~ wherein the target sequence comprises DNA or RNA.

20. **(AMENDED)** A method according to ~~[any one of claims 16-19]~~ **claim 16**, wherein the target sequence is DNA or RNA formed as a result of the presence in the sample of the nucleic acid sequence of interest.

21. **(AMENDED)** A method according to ~~[any one of claims 16-20]~~ **claim 16**, wherein the RNA molecule is detected directly or indirectly by means of a labelled binding partner.

23. **(AMENDED)** A method according to claim 21 ~~[or 22]~~, wherein the labelled binding partner comprises DNA, RNA, LNA, PNA, or any combination thereof.

24. **(AMENDED)** A kit for use in performing ~~[the method of]~~**a method of detecting a nucleic acid sequence of interest,** any one of claims 16-23, comprising a probe molecule in accordance with ~~[any one of claims 1-14]~~ **claim 1**, and packaging means.

25. **(AMENDED)** A kit according to claim 24, further comprising one or more of the following: instructions for performing the method ~~[of any one of claims 16-23]~~; a buffer; a DNA polymerase; an RNA polymerase; deoxyribonucleotide triphosphates; ribonucleotide triphosphates; and a labelled binding partner.

26. A method of detecting a nucleic acid sequence of interest in a sample, the method comprising the steps of: contacting a nucleic acid probe molecule in accordance with ~~[any one of claims 1-14]~~ **claim 1** with a further probe and with a nucleic acid target molecule, which target is the sequence of interest or is formed as a result of the presence in the sample of the sequence of interest; causing the further probe molecule and the target molecule to hybridise adjacent each other to the probe molecule, thereby creating a functional double stranded RNA polymerase promoter; causing RNA synthesis from the RNA polymerase promoter, to create ~~[an]~~ **a** RNA molecule; and detecting directly or indirectly the RNA molecule so produced.

METHOD FOR AMPLIFICATION OF NUCLEIC ACIDS

Field of the Invention

This invention relates to nucleic acid probe molecules, methods involving use of the probe molecules, and kits comprising the probe molecules.

Background of the Invention

A number of nucleic acid amplification processes are disclosed in the prior art. One such process is the polymerase chain reaction (PCR) and is disclosed in US 4683195 and 4683202. The PCR process is well known and widely used but is not suitable for all applications since the method has some drawbacks including the need for adjusting reaction temperatures alternately between intermediate (e.g. 50°C-55°C) and high (e.g. 90°C-95°C) temperatures involving repeated thermal cycling. Also the time scale required for multiple cycles of large temperature transitions to achieve amplification of a nucleic acid sequence and the occurrence of sequence errors in the amplified copies of the nucleic acid sequence is a major disadvantage as errors occur during multiple copying of long sequence tracts. Additionally, detection of the amplified nucleic acid sequence generally requires further processes e.g. agarose gel electrophoresis.

Alternative nucleic acid amplification processes that utilize RNA polymerases are disclosed in WO 88/10315 (Siska Diagnostics), EP 329822 (Cangene) EP 373960 (Siska Diagnostics), US 5,554,516 (Gen-Probe Inc.), WO 89/01050 (Burg *et al*), WO 88/10315 (Gingeras *et al*), and EP 329822 (Organon Teknika), which latter document relates to a technique known as NASBA. These amplification processes describe a cycling reaction comprising of alternate DNA and RNA synthesis. This alternate RNA/DNA synthesis is achieved principally through the annealing of oligonucleotides adjacent to a specific DNA sequence whereby these oligonucleotides comprise a transcriptional promoter. The RNA copies of the specific sequence so produced, or alternatively an input sample comprising a specific RNA sequence (US 5,554,516), are then copied as DNA strands using a nucleic acid primer and the RNA from the resulting DNA:RNA hybrid is either removed by denaturation (WO 88/10315) or removed with RNase H (EP 329822, EP 373960 & US 5,554,516).

Two further amplification processes are disclosed, one thermal and one isothermal, in WO 93/06240. Both the thermal and isothermal versions depend on the hybridisation of two nucleic acid probes of which regions are complementary to the target nucleic acid. Portions of the probes are capable of hybridising to the sequence of interest such that the probes are adjacent or substantially adjacent to one another, so as to enable complementary arm specific sequences of the first and second probes to become annealed to each other. Following annealing, chain extension of one of the probes is achieved by using part of the other probe as a template. Amplification of the extended probe is achieved by one of two means; in the thermal cycling version thermal separation of the extended first probe is carried out to allow hybridisation of a further probe, substantially complementary to part of the newly synthesised sequence of the extended first probe. Extension of the further probe by use of an appropriate polymerase using the extended first probe as a template is achieved. Thermal separation of the extended first and further probe products can act as a template for the extension of further first probe molecules and the extended first probe can act as a template for the extension of other further probe molecules.

In the isothermal version, primer extension of the first probe creates a functional RNA polymerase promoter that in the presence of a relevant RNA polymerase transcribes multiple copies of RNA. The resulting RNA is further amplified as a result of the interaction of complementary DNA oligonucleotides containing further RNA polymerase promoter sequences, whereupon annealing and extension of the RNA on the DNA oligonucleotide leads to a further round of RNA. This cyclical process generates large yields of RNA, detection of which can be achieved by a number of means.

Summary of the Invention

In a first aspect the invention provides a probe molecule comprising single stranded nucleic acid; said probe comprising a single stranded sequence complementary to a target nucleic acid sequence; a single strand of an RNA polymerase promoter sequence; and a blocking moiety adjacent or substantially adjacent to the promoter sequence.

RNA polymerase promoter sequences are well known to those skilled in the art. Preferred promoter sequences are those recognised by bacteriophage polymerases, especially T3, T7 or SP6 polymerase. T3, T7 and SP6 promoter sequences are typically 17 bases long. To be fully functional, the promoter sequence must be present in double stranded form.

An example of a double stranded promoter sequence recognised by T7 polymerase is:

5' GAA ATT AAT ACG ACT CAC TAT A*GG GAG AGA GAG C* 3'
3' CTT TAA TTA TGC TGA GTG ATA T*CC CTC TCT CTC G* 5' (seq ID No. 1)
"-5" sequence promoter "+12" sequence

The promoter sequence is underlined (the significance of the -5 and +12 sequences is explained elsewhere). The promoter sequence recognised by the T7 polymerase may be referred to as the T7 promoter. In fact, several minor variants of the sequence (typically differing by only a single base) are known to be recognised by T7 polymerase, and to act as functional promoters.

It is known in the prior art to provide single stranded probe molecules which comprise one strand of an RNA polymerase promoter. When combined with the complementary strand, a fully functional double stranded promoter is formed, which may then allow for RNA synthesis (transcription) using adjacent sequences downstream of the template strand of the promoter as template. Typically, in the prior art, formation of the double stranded, functional promoter occurs only as a result (directly or indirectly) of the presence of a particular target nucleic acid sequence of interest, such that RNA synthesis is (directly or indirectly) indicative of the presence of the sequence of interest.

However, the inventors have found that such systems may be associated with a relatively high degree of "background" signal (i.e. that RNA synthesis may occur in the absence of the sequence of interest), which can reduce the sensitivity, or reproducibility, of the assay. Surprisingly, the inventors have discovered that the signal: noise ratio can be dramatically improved by inclusion in the single stranded probe molecule of a "blocking moiety" which is not recognised by an RNA polymerase, the blocking moiety being positioned adjacent, or substantially adjacent, to the RNA polymerase promoter sequence.

The term "adjacent" as used in the context of the invention, is intended to indicate that there are no nucleic acid bases between the blocking moiety and the promoter sequence. In practice, it will normally be preferred that the blocking moiety is not directly adjacent to the promoter (i.e. is placed "substantially adjacent"). The term "substantially adjacent" as

used in the context of the invention, is intended to indicate that there are between 1 and 50 nucleic acid bases between the blocking moiety and the promoter sequence, preferably between 1 and 40, more preferably between 1 and 30, and most preferably between 1 and 20 bases. In particular, the inventors have found that a spacing of about 12-15 bases between the promoter and the blocking moiety appears to confer optimum results, especially where blocking moiety comprises an alkylene moiety.

The accepted numbering convention is that, for the template strand, the most 3' base of the promoter (assuming the promoter sequence consists of 17 bases) is indicated as position -17. Accordingly, an adjacent blocking moiety, positioned 3' of the promoter on the template strand, would be at position -18, and a "substantially adjacent" blocking moiety could be positioned anywhere between position -19 to -68, with preferred positioning being between -19 to –38, more especially between position –22 and –35.

It will generally be preferred, where the nucleic acid probe molecule comprises the template strand of the RNA promoter, that the blocking moiety will be positioned 3' of the promoter. Conversely, where the probe molecule comprises the non-template strand of the promoter it will be preferred to position the blocking moiety 5' of the promoter.

It will normally be preferred that the probe molecule comprises the template strand of the RNA promoter sequence, but this is not essential.

As mentioned above, it will normally be preferred that there are several nucleic acid bases between the promoter sequence and the blocking moiety. Preferably the sequence of at least some of the intervening nucleic acid bases (especially the sequence immediately adjacent to the promoter) will be arranged so as to optimise promoter activity of the functional double stranded promoter. In particular, a "-5" sequence is preferably present. The -5 sequence is a sequence of 5 bases immediately 5' of the template strand of the promoter sequence, which has been found to enhance promoter activity. The optimum sequence of bases constituting the -5 sequence tends to vary for each polymerase. For T7 polymerase, the optimum -5 sequence is that shown in bold letters above on page 3.

In principle, the blocking moiety included in the probe may be any entity which is not recognised as suitable template by an RNA polymerase. In practice, it is desirable that the blocking moiety is capable of insertion in synthetic oligonucleotides by incorporation of appropriate precursors (e.g. phosphoramidites) during *in vitro* synthesis of the oligonucleotide. Desirably the blocking moiety is unable to form base pairs with nucleic acid, and is advantageously located other than at the extreme 5' or 3' terminus. Preferred blocking moieties include alkylene, alkanol and alkyl residues, especially C_2-C_{20} alkyl, alkanol or alkylene, more preferably C_3-C_{10} alkyl, alkanol or alkylene. Particular examples include octanediol, propanediol, hexaethylene glycol, and propyl residues.

In some preferred embodiments, the nucleic acid probe molecule comprises a plurality of blocking moieties, which may be positioned adjacent each other in the probe, or may be separated by intervening nucleic acid bases. The plurality of blocking moieties may be of the same chemical entity, or may be different.

The probe molecule of the invention may comprise DNA, RNA, PNA (peptide nucleic acid) or LNA (locked nucleic acid), or any combination thereof. Generally, the single stranded promoter sequence will normally substantially consist of DNA, although a few (e.g. 1-3) bases may be of, for example, RNA, PNA or LNA and still allow for the formation of a functional promoter when the promoter sequence is hybridised with a complementary DNA sequence.

PNA is a synthetic nucleic acid analogue in which the sugar/phosphate backbone is replaced by a peptide-linked chain (typically of repeated N-(2-aminoethyl)-glycine units), to which the bases are joined by methylene carbonyl linkages. PNA/DNA hybrids have high Tm values compared to double stranded DNA molecules, since in DNA the highly negatively-charged phosphate backbone causes electrostatic repulsion between the respective strands, whilst the backbone of PNA is uncharged. Another characteristic of PNA is that a single base mis-match is, relatively speaking, more destabilizing than single base mis-match in heteroduplex DNA. Accordingly, PNA is useful to include in probes for use in the present invention, as the resulting probes have greater specificity than probes consisting entirely of DNA. Synthesis and uses of PNA have been disclosed by, for

example, Orum *et al*, (1993 Nucl. Acids Res. **21**, 5332); Egholm *et al*, (1992 J. Am. Chem. Soc. **114**, 1895); and Egholm *et al*, (1993 Nature **365**, 566).

LNA is a synthetic nucleic acid analogue, incorporating "internally bridged" nucleoside analogues. Synthesis of LNA, and properties thereof, have been described by a number of authors: Nielson *et al*, (1997 J. Chem. Soc. Perkin Trans. 1, 3423); Koshkin *et al*, (1998 Tetrahedron Letters **39**, 4381); Singh & Wengel (1998 Chem. Commun. 1247); and Singh *et al*, (1998 Chem. Commun. 455). As with PNA, LNA exhibits greater thermal stability when paired with DNA, than do conventional DNA/DNA heteroduplexes. However, LNA can be synthesised on conventional nucleic acid synthesising machines, whereas PNA cannot.

In addition to non-conventional nucleic acids such as LNA and PNA, the probe molecule of the invention may also comprise, if desired, modified nucleic acid bases, such as inosine and the like. In particular, it may be preferred to provide the probe with a modified 3' end, such as a dideoxynucleotide or other modified base known to those skilled in the art, so as to prevent chain extension of the probe by DNA polymerases.

It is preferred that at least that portion of the probe molecule which hybridises to a nucleic acid target comprises PNA and/or LNA. The target to which the probe molecule hybridises will typically comprise RNA and/or DNA.

The target to which the probe hybridises may be the actual sequence of interest (e.g. a human or other gene, which may be a marker of genetic or infectious disease), present in a sample to be investigated. Alternatively, the presence in a sample of the sequence of interest may lead (directly or indirectly) to the presence of a further nucleic acid sequence, which is the target to which the nucleic acid probe hybridises.

For example, the presence in a sample (such as a sample of body fluid from a human or animal subject, or from an environmental sample) of a sequence of interest may lead to the synthesis of a further RNA or DNA target sequence, to which the nucleic acid probe of the

invention hybridises, the target nucleic acid sequence being formed by an assay method such as that disclosed in WO 93/06240, WO 99/37805 or WO 99/37806, for example.

Thus in a particular embodiment, a nucleic acid probe molecule in accordance with the invention may comprise (in the direction 5' to 3'); a template portion to be transcribed into RNA; (optionally) a +12" portion (a sequence of 12 bases immediately adjacent to the 5' end of the promoter, which serves to enhance promoter activity in a manner similar to the -5 sequence: a particular example of a +12 sequence for the T7 polymerase is shown previously on page 3 in italic type; a promoter sequence; (optionally) a -5 sequence; and a portion complementary to the target; with the one or more blocking moieties typically positioned 3' of the -5 sequence, generally within the target-complementary portion of the probe.

Desirably the sequence of the probe molecule will be selected so as to hybridise near the 3' end region of the target such that the 3' end of the target may be extended by an appropriate DNA polymerase, in the presence of deoxyribonucleotide triphosphates, using the probe as template. This extension of the target creates a complementary strand for the single stranded promoter sequence present in the probe, such that a double stranded, functional RNA polymerase promoter is formed, which can then cause the formation of RNA (in the presence of an appropriate RNA polymerase and ribonucleotide triphosphates). This RNA species may be detected directly, or may in turn be hybridised to a further probe (comprising a single stranded RNA promoter) and extended, thereby creating a further double stranded RNA polymerase promoter which leads to synthesis of a further RNA species. If desired the sequences of the probes may be selected such that the sequence of the further RNA species matches (at least in part) that of the original target, such that the further RNA species may hybridise to the original probe molecule, restarting the process and leading to an amplification cycle. Such a cycle may form the basis of an extremely sensitive detection assay.

Alternatively, the RNA species may be the subject of a "stepped" amplification, in which each RNA species produced hybridises to a respective probe, and can be extended to create a double stranded promoter, leading to the synthesis of a further RNA species. The

number of amplification steps may be as many as desired, leading (eventually) to considerable amplification of the original signal but without undergoing any cycling.

Amplification cycles and steps of this sort are disclosed in the prior art, but the prior art does not disclose the use of probes comprising blocking moieties which might be expected to interfere with the process. Conversely, and unexpectedly, the inventors have found that use of probes in accordance with the invention reduces the background signal (i.e. amount of RNA produced in the absence of target), thereby improving the assay system.

It will be apparent to those skilled in the art that probe molecules in accordance with the invention may be usefully employed in an assay method of detecting a nucleic acid sequence of interest in a sample.

Thus, in a second aspect, the invention provides a method of detecting a nucleic acid sequence of interest in a sample, the method comprising the steps of: contacting a nucleic acid probe molecule in accordance with the first aspect of the invention defined above with a nucleic acid target molecule, which target is the sequence of interest or is formed as a result of the presence in the sample of the sequence of interest; causing extension of the 3' end of the target using the probe as a template, thereby creating a functional double stranded RNA polymerase promoter; causing RNA synthesis from the RNA polymerase promoter, to create an RNA molecule; and detecting directly or indirectly the RNA molecule so produced.

The step of causing extension of the 3' end of the target will conveniently be performed by addition of deoxyribonucleotide triphosphates in the presence of a DNA polymerase (such as Klenow fragment, or *Bst* polymerase) and in a suitable reaction buffer. Suitable reaction conditions are well known to those skilled in the art.

Typically the blocking moiety, in addition to blocking RNA polymerase, will tend also to reduce non-specific interactions involving DNA polymerase. Accordingly it will normally be preferred that the probe hybridises to the target in such a manner that the DNA polymerase will not need to extend the target past a blocking moiety (which will therefore

be present in that part of the probe which hybridises to the target, although it will be apparent that the blocking moiety itself will tend not to be capable of base pairing, and so will not be base paired with the target).

The step of causing RNA synthesis will typically be effected by causing to be present an RNA polymerase which recognises the RNA promoter, together with suitable ribonucleotide triphosphates. Preferred polymerases are T3,T7 and SP6 polymerase.

The RNA molecule produced may itself be detected (i.e. "direct detection") or, as explained above, may be used to form one or more further RNA molecules (conveniently by means of a cycling or stepped amplification procedure) which may be detected ("indirect detection") by any desired means. A great number of detection methods are known to those skilled in the art. Preferably detection will involve use of a labelled binding partner (e.g. a PNA, LNA, DNA or RNA sequence which hybridises to the original or further RNA molecules), typically being labelled with an enzyme label (horseradish peroxidase or alkaline phosphatase are preferred) or a fluorophore, a radio label or a biochemical label.

For example, newly-synthesised RNA could be detected in a conventional manner (e.g. by gel electrophoresis), with or without incorporation of labelled bases during the synthesis.

Alternatively, for example, newly-synthesised RNA could be captured at a solid surface (e.g. on a bead, or in a microtitre plate), and the captured molecule detected by hybridisation with a labelled nucleic acid probe (e.g. radio-labelled, or more preferably labelled with an enzyme, chromophore, fluorophore and the like). Preferred enzyme labels include horseradish peroxidase and alkaline phosphatae.

One preferred detection method involves the use of molecular beacons or the techniques of fluorescence resonance energy transfer ("FRET"), delayed fluorescence energy transfer ("DEFRET") or homogeneous time-resolved fluorescence ("HTRF"). Molecular beacons are molecules with which a fluorescence signal may or may not be generated, depending on the confirmation of the molecule. Typically, one part of the molecule will comprise a

fluorophore, and another part of the molecule will comprise a "quencher" to quench fluorescence from the the fluorophore. Thus, when the conformation of the molecule is such that the fluorophore and quencher are in close proximity, the molecular beacon does not fluoresce, but when the fluorophore and the quencher are relatively widely-separated, the molecule does fluoresce. The molecular beacon conveniently comprises a nucleic acid molecule labelled with an appropriate fluorophore and quencher.

One manner in which the conformation of the molecular beacon can be altered is by hybridisation to a nucleic acid, for example inducing looping out of parts of the molecular beacon. Alternatively, the molecular beacon may initially be in a hair-pin type structure (stabilised by self-complementary base-pairing), which structure is altered by hybridisation, or by cleavage by an enzyme or ribozyme.

FRET (Fluorescence Resonance Energy Transfer) occurs when a fluorescent donor molecule transfers energy via a nonradiative dipole-dipole interaction to an acceptor molecule. Upon energy transfer, which depends on the R^{-6} distance between the donor and acceptor, the donor's lifetime and quantum yield are reduced and the acceptor fluorescence is increased or sensitised.

Another approach (DEFRET, Delayed Fluorescence Energy Transfer) has been to exploit the unique properties of certain metal ions (Lanthanides e.g. Europium) that can exhibit efficient long lived emission when raised to their excited states (excitation = 337 nm, emission = 620 nm). The advantage of such long lived emission is the ability to use time resolved (TR) techniques in which measurement of the emission is started after an initial pause, so allowing all the background fluorescence and light scattering to dissipate. Cy5 (Amersham Pharmacia) (excitation = 620 nm, emission = 665 nm) can be used as the DEFRET partner.

HTRF (see WO 92/01224; US 5,534,622) occurs where a donor (e.g. Europium) is encapsulated in a protective cage (cryptate) and attached to the 5' end of an oligomer. The acceptor molecule that has been developed for this system is a protein fluorophore, called

XL665. This molecule is linked to the 3' end of a second probe. This system has been developed by Packard.

In another embodiment, the newly-synthesised RNA, before or after amplification, results in formation of a ribozyme, which can be detected by cleavage of a particular nucleic acid substrate sequence (e.g. cleavage of a fluorophore/quencher dual-labelled oligonucleotide).

In a third aspect the invention provides a kit for use in the method of the second aspect, the kit comprising one or more probe molecules in accordance with the first aspect of the invention, and packaging means.

The kit may optionally comprise one or more of the following: instructions for performing the method of the invention; buffers; a DNA polymerase; an RNA polymerase; dNTPs; rNTPs; and a labelled binding partner.

The invention will now be further described by way of illustrative example and with reference to the accompanying drawings, wherein:

Figure 1 is a schematic representation of a blocking moiety, hexaethylene glycol, incorporated into a DNA probe in accordance with the invention;

Figures 2, 3 and 6, are schematic representations of nucleic acid probes in accordance with the invention, Figures 2 and 6 illustrating preferred embodiments;

Figures 4 and 5 are schematic representations of a pair of nucleic acid probes in accordance with the invention, which may be used in an amplification cycle;

Figure 7 is a schematic representation of an amplification cycle utilising the probes illustrated in Figures 4 and 5;

Figures 8-12 and 14-16 are bar charts comparing the yield (in femtomoles of RNA) for various reactions using probes in accordance with the invention comprising a blocking moiety, with those comprising conventional probes without a blocking moiety; and

Figures 13a and 13b are schematic representations of reaction schemes.

Example 1

This example demonstrated the use of Hexaethylene glycol (Hex) linkers as blockers of RNA polymerase single strand read through and other non-specific side reactions caused by DNA polymerase. Hex linkers were incorporated between bases –52/-53, -41/-42 and –29/-30. In addition, Hex linkers were incorporated at all three positions.

1.1 Preparation of oligonucleotides

All oligonucleotide probes were synthesised by phosphoramidite chemistry using an Applied Biosystems 380A synthesiser according to the manufacturer's instructions. Hex incorporation was accomplished by reaction of the growing chain with 18-dimethoxytrityl hexaethylene glycol, 1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. Biotinylation of oligonucleotide probes was achieved by incorporation of a biotin phosphoramidite. Oligonucleotides functionalised with alkaline phosphatase were prepared using the manufacturer's proprietary method (Oswel). All oligonucleotides were HPLC purified using standard techniques.

1.2 Preparation of RNA

RNA was prepared using 1 pmol of probes 1 and 2, T7 RNA polymerase buffer (Promega) (40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl); T7 RNA polymerase (Promega) (25 units) and 2 µl rNTP mix (Amersham Pharmacia Biotech) (20 mM of each NTP: adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP) uridine 5'-triphosphate (UTP)). The reaction volume was made up to 20 µl with RNase-free distilled water. The mixture was incubated at 37°C for 3 hours. DNA (probes 1 and 2) was removed from the assay mixture using RNase-free DNase (Ambion) (3 units DNase added per 20 µl assay mix, incubated at 37 °C for 10 minutes and 90 °C for 3 minutes).

1.3 Synthesis of RNA off hybridised oligonucleotide

Hybridisation was achieved in an assay mixture that contained 10 fmol of linear DNA template (probe 3, 4, 5, 6 or 7) and 10 fmol of RNA complementary to the 3' end of the linear template (prepared as described above); Bst DNA polymerase (New England Biolabs) (3' → 5' exo-, 4 units); 1 µl dNTP mix (Amersham Pharmacia Biotech) (2.5 mM of each dNTP: 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxythymidine 5'-triphosphate (dTTP), 2'-deoxyguanosine 5'-triphosphate (dGTP) and 2'-deoxycytidine 5'-triphosphate (dCTP)); T7 RNA polymerase buffer (Promega); T7 RNA polymerase (25 units) and 2 µl rNTP mix (Amersham Pharmacia Biotech). The reaction volume was made up to 20 µl with RNase-free distilled water. Control reactions contained linear template without complementary RNA. The mixture was incubated at 41°C for 3 hours.

1.4 Capture and detection of synthesised RNA

5 µl of assay sample was added to 145 µl hybridisation buffer (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 20 mM EDTA and 0.1% BSA) containing 0.9 pmol biotinylated capture oligonucleotide (specific for the RNA to be detected) and 6 pmol specific alkaline phosphatase oligonucleotide in streptavidin coated wells. Incubation (60 minutes at room temperature, shaking at 300 rpm) allowed the RNA to be immobilised on the wells via the biotinylated capture probe and annealed to the detection probe. Unbound material was removed from the wells by washing four times with TBS/0.1% Tween-20, then once with alkaline phosphatase substrate buffer (Boehringer Mannheim). Finally, alkaline phosphatase substrate buffer containing 4-nitrophenyl phosphate (5mg/ml) was added to each well. The plate was incubated at 37°C in a Labsystems EIA plate reader and readings were taken at 405 nm every 2 minutes. Results are presented in Figure 8 in the form of a bar chart. In Figure 8, the pairs of columns show the amount of RNA produced in various reactions (the left hand column of each pair shows the result for the full reaction, the right hand column indicates the amount of RNA produced in the control reactions i.e. indicates the background signal). From left to right, the results are those obtained using a probe with a) no Hex; b) Hex between base -53/-54 (assuming the most 3' base of the promoter sequence is at position -17); c) hex between -41/-42; d) hex between base -29/-20; and e) probe with three hexs, one each between bases -53/-54, -41/-42 and -29/-30. The

numbers above the columns indicate the amplification factor (upper number) and signal: noise ratio (lower number) respectively. From the results it can be seen that background RNA was generated when the linear DNA templates only were reacted with T7 RNA polymerase and *Bst* DNA polymerase. This becomes problematic in an amplification assay since the background would be amplified resulting in poor signal:noise. Introducing Hexaethylene glycol linkers in the region of the DNA template where the DNA or RNA primer hybridised resulted in much less background. The location of this linker with respect to the distance from the T7 promoter was important in controlling the amount by which the background was reduced. In addition, the single Hexaethylene glycol linker between bases –29/-30 caused the same reduction in background as three Hexaethylene glycol linkers between bases –53/-54, -41/-42 and 29/30.

1.5 List of oligonucleotides (H = Hex linker)

Probe 1 Seq ID No. 2

5'TGCCTCCTTGTCTCCGTTCTGGATATCACCCGATGTGTCTCCCTATAGTGAGTC GTATTAATTTC 3'

Probe 2 Seq ID No. 3

5'GAAATTAATACGACTCACTATA 3'

Sequence of transcribed RNA Seq ID No. 4

5'GGGAGACACAUCGGGUGAUAUCCAGAACGGAGACAAGGAGGCA 3'

Probe 3 (Template, No Hex) Seq ID No. 5

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTG AGTCGTATTAATTTCTGCCTCCTTGTCTCCGTTCTGGATATCACCCGATGTGTCT CCC 3' phosphate

Probe 4 (Template, as Seq ID No. 5 but with Hex between bases –53/-54)

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGAGT CGTATTAATTTCTGCCTCCTTGTCTCCGTTCTGGATATCACCCHGATGTGTCTCC C 3' phosphate

Probe 5 (Template, as Seq ID No. 5 but with Hex between bases –41/–42)
5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGAGT
CGTATTAATTTCTGCCTCCTTGTCTCCGTTCHTGGATATCACCCGATGTGTCTCC
C 3' phosphate

Probe 6 (Template, as Seq ID No. 5 but with Hex between bases –29/–30)
5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGAGT
CGTATTAATTTCTGCCTCCHTTGTCTCCGTTCTGGATATCACCCGATGTGTCTCC
C 3' phosphate

Probe 7 (Template, as Seq ID No. 5 but with Hex in all 3 positions)
5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGAGT
CGTATTAATTTCTGCCTCCHTTGTCTCCGTTCHTGGATATCACCCHGATGTGTCT
CCC 3' phosphate

Capture Probe Seq ID No. 6
5'TCTGCTGCCTGCTTGTCTGCGTTCT 3' (5' biotinylated)

Detection probe Seq ID No. 7
5'GGATATCACCCG3' (3' alkaline phosphatase labelled)

The results of this experiment suggested (see Figure 8) that the signal : noise ratio was significantly affected by the position and number of blocking Hex linkers included in the DNA probes. To investigate further, the experiment was repeated, with essentially identical probe molecules but including some further probes comprising hex residues between bases –24/–25, -27/-28, -31/-32 or –33/–34. The results are shown in Figure 9, which uses the same notation as Figure 8.

These results showed that a single Hexaethylene glycol linker between bases –31/–32 resulted in a high yield of RNA transcription with low background. Three Hexaethylene

glycol linkers between bases –53/-54, -41/-42 and –29/-30 resulted in no detectable background but a lower signal.

Example 2

This example demonstrated the use of Hex linkers in the cycled amplification of RNA. Two DNA templates were used. The first DNA template annealed RNA1, followed by extension by DNA polymerase and transcription of RNA2. The second template annealed RNA2 followed by extension by DNA polymerase and transcription of RNA1. Reactions were performed using two DNA templates without Hex linkers and with two DNA templates each containing three Hex linkers.

2.1 Preparation of oligonucleotides

All oligonucleotide probes were synthesised and purified as described in Example 1.

2.2 Preparation of RNA

RNA1 was prepared using 1 pmol of probes 1 and 2, T7 RNA polymerase buffer (Promega);T7 RNA polymerase (Promega) (25 units) and 2 µl rNTP mix (Amersham Pharmacia Biotech). The reaction volume was made up to 20 µl with RNase-free distilled water. The mixture was incubated at 37°C for 3 hours. DNA (probes 1 and 2) was removed from the assay mixture using RNase-free Dnase (Ambion) (3 units DNase added per 20 µl assay mix, incubated at 37°C for 10 minutes and 90 °C for 3 minutes).

2.3 Synthesis of RNA off hybridised oligonucleotide

The reaction mixture contained 1 fmol of probe 3 (no Hexs) or probe 4 (three Hexs) and 1 fmol of RNA prepared as in 2.2 above. This RNA was complementary to the 3' end of probes 3 and 4; *Bst* DNA polymerase (New England Biolabs) (3' → 5' exo-minus, 4 units); 1 µl dNTP mix (Amersham Pharmacia Biotech); T7 RNA polymerase buffer; T7 RNA polymerase (25 units) and 2 µl rNTP mix. The reaction volume was made up to 20 µl with RNase-free distilled water. The mixture was incubated at 41°C for 1 or 2 hours. At these intervals 100 fmol of probe 5 (no Hexs) or probe 6 (three Hexs) was added to the reactions containing probe 3 or probe 4, respectively. The mixture was reincubated at

41°C for a total reaction time of 3 hours. Control reactions contained the same probes used in the full reactions but without complementary RNA.

2.4 Capture and detection of synthesised RNA

5 µl of assay sample from 2.3 was processed as described in Example 1.4. Results are presented in Figure 10. Figure 10 shows the RNA yield for test and control reactions (in pairs) for DNA template probes without hex blocking moieties (two left hand pairs of columns) or probes comprising hex moieties (right hand pairs of columns). For each set of probes, the experiment was performed with second DNA template (for the cycling amplification reaction) added after 1 hour or 2 hours' incubation as indicated. The importance of Hexaethylene glycol linkers was demonstrated when performing cycled amplification with two DNA templates. If DNA templates were used without chemical linkers then although amplification did occur, the yield of RNA was poor and the signal: noise was low. With chemical linkers, however, the signal was increased and the signal: noise was also improved. This improves the amplification process and would lead to increased sensitivity in nucleic acid amplification assay.

2.5 List of oligonucleotides (H = Hex linker)

Probe 1 Seq ID No. 8
5'TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAATCGTCAGTCCCTATAGTGA
GTCGTATTAATTTC 3'

Probe 2 as Seq ID No. 3, Example 1

Sequence of transcribed RNA Seq ID No. 9
5'GGGACUGACGAUUCGGGUGAUAUCCAGAACGCAGACAAGCAGGCA 3'

Probe 3 (No Hexs) Seq ID No. 10
5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGAGT
CGTATTAATTTCTGCCTGCTTGTCTGCGTTCTGGATATCACCCGAATCGTCAGTC
CC 3' phosphate

Probe 4 (as Seq ID No. 10 but with Hexs between bases -29/-30, -41/-42 and -53/-54)
5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTG
AGTCGTATTAATTTCTGCCTGCHTTGTCTGCGTTCHTGGATATCACCCHGAATC
GTCAGTCCC 3' phosphate

Probe 5 Seq ID No. 11
5'TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAATCGTCAGTCCCTATAGTGA
GTCGTATTAATTTCTCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCT
TCC 3' phosphate

Probe 6 (as Seq ID No. 11 but with Hexs between bases -28/-29, -40/-41 and -53/-54)
5'TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAATCGTCAGTCCCTATAGTGA
GTCGTATTAATTTCTCGTCTHTCCGGTCTCTCCHTCTCAAGCCTCAHGCGTTCTC
TCTTCC 3' phosphate

Capture Probe as Seq ID No. 6, Example 1

Detection probe as Seq ID No. 7, Example 1

Example 3

This example demonstrated that a Hexaethylene glycol (Hex) linker located between bases -31/-32 caused a reduction in background. Using *Bst*, Klenow or Phi29 DNA polymerase for extension, a Hex linker lowered the background using three different DNA templates.

3.1 Preparation of oligonucleotides

All oligonucleotide probes were synthesised and purified as described previously.

3.2 Preparation of RNA

RNA was prepared using T7 RNA polymerase buffer (Promega); T7 RNA polymerase (Promega) (25 units) and 2 µl rNTP mix (Amersham Pharmacia Biotech). The reaction volume was made up to 20 µl with RNase-free distilled water. The mixture was incubated

at 37°C for 3 hours. DNA was removed from the assay mixture using RNase-free Dnase (Ambion) (3 units DNase added per 20 µl assay mix, incubated at 37 °C for 10 minutes and 90 °C for 3 minutes). RNA1A was transcribed using 1 pmol probes 1A and 2A; RNA2A was prepared using 1 pmol probes 2A and 3A; RNA3A was prepared using 1 pmol probes 2 A and 4A.

3.3 Synthesis of RNA off hybridised oligonucleotide

Hybridisation was achieved in an assay mixture that contained 10 fmol of linear DNA template and 10 fmol of RNA complementary to the 3' end of the linear template; *Bst* (New England Biolabs) (3' → 5' exo-, 4 units), Klenow (New England Biolabs) (3' → 5' exo-, 4 units) or Phi29 (Amersham Pharmacia Biotech) DNA polymerase (3' → 5' exo-, 1 unit); 1 µl dNTP mix (Amersham Pharmacia Biotech); T7 RNA polymerase buffer; T7 RNA polymerase (25 units) and 2 µl rNTP mix (Amersham Pharmacia Biotech). The reaction volume was made up to 20 µl with RNase-free distilled water. Control reactions contained linear template without complementary RNA. The mixture was incubated at 41°C for 3 hours.

3.4 Capture and detection of synthesised RNA

5µl of assay sample was added to 145 µl hybridisation buffer (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 20 mM EDTA and 0.1% BSA) containing 0.9 pmol biotinylated capture oligonucleotide (Capture probe 1 was used to detect the transcripts from Probes 9A and 10A; Capture probe 2 was used to detect the transcripts from Probes 5A, 6A, 7A and 8A) and 6 pmol alkaline phosphatase oligonucleotide (Detection Probe 1 was used to detect the transcripts from Probes 9A and 10A; Detection Probe 2 was used to detect the transcripts from Probes 5A, 6A, 7A and 8A) in streptavidin coated wells. Microtitre plates were then processed as described in previous examples. Results are presented in Figures 11a, 11b and 11c. Figures 11a-11c use the same general notation as used in Figures 8-10. In Figures 11a to 11c there are 3 pairs of columns (i), (ii) and (iii), which indicate results obtained using Bst, Klenow or Phi 29 polymerase respectively. For each polymerase, the left hand pairs of columns indicates the results obtained using probes without hex moieties, and the right hand pairs of columns are results obtained using hex-containing probes. Figure 11a shows the results of reactions using RNA 1A and probes 5A (no hex) or 6A

(with hex). Figure 11b shows the results of reactions using RNA2A and probes 7A (no hex) or 8A (with hex). Figure 11c shows the results of reactions using RNA 3A and probes 9A (no hex) or 10A (with hex). This work showed that the same reduction in background was observed when DNA templates containing a Hexaethylene glycol linker were incubated with DNA polymerases other than *Bst* DNA polymerase. Also, a Hexaethylene glycol linker located between bases -3-/-32 reduced the background in three different DNA template sequences. However, the amount of RNA transcribed was dependent on the sequence of the DNA template used.

3.5 List of oligonucleotides (H = Hex linker)

Probe 1A – as Seq ID No. 2, Example 1

Probe 2A – as Seq ID No. 3, Example 1

RNA1A – as Seq ID No. 4, Example 1

Probe 3A Seq ID No. 12

5'TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCTCGCTTCCTATAGT
GAGTCGTATTAATTTCTCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTGTCT
CTTCC3'

RNA2A Seq ID No. 13

5'GGAAGCGAGAACUCGGGUGAUAUCCAGAACGCAGACAAGCAGGCA 3'

Probe 4A Seq ID No. 14

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTGTCTCTTCCTATAGTG
AGTCGTATTAATTTCTGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCTCG
CTTCC3'

RNA3A Seq ID No. 15

5'GGAAGAGACAACGCUGAGGCUUGAGAGGAGAGACCGGAAGACGA3'

Probe 5A as Seq ID No. 5, Example 1

Probe 6A (as Seq ID No. 5 but with Hex between bases -31/-32)
5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTCTCTCTTCCTATAGTGAGT
CGTATTAATTTCTGCCTCCTTHGTCTCCGTTCTGGATATCACCCGATGTGTCTCC
C3'

Probe 7A No Hex Seq ID No. 16
5' TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTGTCTCTTCCTATAGTG
AGTCGTATTAATTTCTGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCTCG
CTTCC 3'

Probe 8A (as Seq ID No. 16 but with Hex between bases -31/-32)
5' TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTGTCTCTTCCTATAGTG
AGTCGTATTAATTTCTGCCTGCTTHGTCTGCGTTCTGGATATCACCCGAGTTCTC
GCTTCC 3'

Probe 9A No Hex (as Seq ID No. 12)
5' TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCTCGCTTCCTATAGT
GAGTCGTATTAATTTCTCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGTTGTCT
CTTCC 3'

Probe 10A (as Seq ID No. 12 but with Hex between bases -31/-32)
5'TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCTCGCTTCCTATAGTGA
GTCGTATTAATTTCTCGTCTTCCHGGTCTCTCCTCTCAAGCCTCAGCGTTGTCTC
TTCC 3'

Capture Probe 1 as Seq ID No. 6, Example 1

Detection probe 1 as Seq ID No. 7, Example 1

Capture Probe 2 Seq ID No. 17

5' TCTGCTCGTCTTCCGGTCTCTCCTC 3' (5' biotinylated)

Detection probe 2 Seq ID No. 18

5' TCAAGCCTCAGC 3' (3' alkaline phosphatase)

Example 4

This example demonstrated that different chemical linkers could be used as blockers of RNA polymerase single strand read through and other non-specific side reactions caused by nucleic acid polymerases. In all cases the chemical linker to be tested was positioned between bases -31/-32.

4.1 Preparation of oligonucleotides

All oligonucleotide probes were synthesised as described previously. Octanediol (Oct) was incorporated by reaction of the growing chain with 8-dimethoxytrityl octanediol, 1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. Propanediol (Pro) was incorporated by reaction of the growing chain with 3-dimethoxytrityl propanediol, 1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. All oligonucleotides were HPLC purified using standard techniques.

4.2 Preparation of RNA

RNA1 was prepared using 100 fmol of probes 1 and 2, T7 RNA polymerase buffer (Promega); T7 RNA polymerase (Promega) (25 units) and 2 µl rNTP mix (Amersham Pharmacia Biotech). The reaction volume was made up to 20 µl with RNase-free distilled water. The mixture was incubated at 37°C for 3 hours.

4.3 Synthesis of RNA off hybridised oligonucleotide

The reaction mixture contained 10 fmol of probe 3, probe 4 or probe 5 and 10 fmol of RNA1 transcribed using probes 1 and 2. This RNA was complementary to the 3' end of probes 3, 4 or 5; *Bst* DNA polymerase (New England Biolabs) (3' → 5' exo-minus, 4

units); 1 µl dNTP mix (Amersham Pharmacia Biotech); T7 RNA polymerase buffer; T7 RNA polymerase (25 units) and 2 µl rNTP mix (Amersham Phrmacia Biotech). The reaction volume was made up to 20 µl with RNase-free distilled water. The mixture was incubated at 37 °C for 3 hours.

4.4 Capture and detection of synthesised RNA

10 µl of assay sample was processed as described in Example 1.4. The results are shown in Figure 12. Figure 12 shows the results obtained using probes for reactions, the probes being identical but for the type of blocking moiety present (hex, octanediol, or propanediol). Results for duplicate experiments are shown. Of the three chemical linkers tested between bases –31/-32, all resulted in the same level of background control. Therefore any of these three linkers could be used with the same effect.

4.5 List of oligonucleotides

Probe 1 Seq ID No. 19

5' TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGCTCTCTCTCCCTATAGTG AGTCGTATTAATTTC 3'

Probe 2 – as Seq ID No. 3, Example 1

Probe 3 (**H** = Hexaethylene glycol) Seq ID No. 20

5' TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCTCGCTTCCTATAGT GAGTCGTATTAATTTCTCGTCTTCC**H**GGTCTCTCCTCTCAAGCCTCAGCGCTCTC TCTCCC 3'

Probe 4 (as Seq ID No. 20 but with **O** = Octanediol)

5' TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCTCGCTTCCTATAGT GAGTCGTATTAATTTCTCGTCTTCC**O**GGTCTCTCCTCTCAAGCCTCAGCGCTCTC TCTCCC 3'

Probe 5 (as Seq ID No. 20 but with **P** = Propanediol)

5' TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCTCGCTTCCTATAGT GAGTCGTATTAATTTCTCGTCTTCCPGGTCTCTCCTCTCAAGCCTCAGCGCTCTC TCTCCC 3'

Sequence of transcribed RNA – as Seq ID No. 13, Example 3

Capture Probe – as Seq ID No. 6, Example 1

Detection probe – as Seq ID No. 7, Example 1

Example 5

This experiment was to establish how T7 RNA polymerase and *Bst* DNA polymerase interacted with a Hex linker. These experiments were performed as shown in Figures 13a and 13b.

These experiments showed that when reactions were performed with a Hex linker positioned downstream of the T7 promoter (Figure 13a), no RNA signal was detected. This occurred with both transcription (Figure 14a) and extension plus transcription (Figure 14b) reactions. Control reactions without a Hex linker (probe 3) resulted in an RNA signal being detected. Also, when a DNA primer was used that did not overlap a Hex linker positioned upstream of the T7 promoter (probe 7; Figure 13b), no RNA signal was detected (Figure 15). The control reaction with the DNA primer overlapping the Hex linker (probe 5) did result in an RNA signal. These results indicated that: (1) transcription by T7 RNA polymerase was terminated when a Hex linker was located in the template to be transcribed, downstream of the T7 promoter; (2) extension and transcription reactions failed when the 3' end of a DNA primer was positioned upstream of a Hex linker in the template to be transcribed.

5.1 Preparation of oligonucleotides

All oligonucleotide probes were synthesised and purified as described previously.

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5.2 Preparation of RNA

RNA1 was prepared using 100 fmol of probes 1 and 2, T7 RNA polymerase buffer; T7 RNA polymerase (25 units) and 2 µl rNTP mix (Amersham Pharmacia Biotech). The reaction volume was made up to 20 µl with RNase-free distilled water. The mixture was incubated at 37°C for 3 hours.

5.3 Synthesis of RNA by transcription

Reaction mixtures contained 10 fmol of probe 2 and probe 3 or 10 fmol of probe 2 and probe 4. Probe 2 was complementary to the -5 and T7 promoter sequences of probes 3 and 4 thereby creating a double stranded T7 promoter. Reactions contained T7 RNA polymerase buffer (Promega); T7 RNA polymerase (Promega) (25 units) and 2 µl rNTP mix (Amersham Pharmacia Biotech). The reaction volume was made up to 20 µl with RNase-free distilled water. The mixture was incubated at 37 °C for 3 hours.

5.4 Synthesis of RNA off hybridised RNA or DNA primers

Reaction mixtures contained 10 fmol of RNA1 and probe 3 or 10 fmol RNA1 and probe 4; 10 fmol probe 5 and probe 6 or 10 fmol probe 7 and probe 6; 10 fmol probe 5 and probe 8 or 10 fmol probe 7 and probe 8. The RNA or DNA primers (RNA1, probe 5 or probe 7) were complementary to the 3' ends of probes 3, 4, 6 and 8. Each reaction contained Bst DNA polymerase (New England Biolabs) (3' → 5' exo-minus, 4 units); 1 µl dNTP mix (Amersham Pharmacia Biotech); T7 RNA polymerase buffer; T7 RNA polymerase (25 units) and 2 µl rNTP mix. The reaction volume was made up to 20 µl with RNase-free distilled water. The mixture was incubated at 37 °C for 3 hours.

5.5 Capture and detection of synthesised RNA

An aliquot of the assay sample was processed as described in preceding examples. RNA transcripts from probes 3 and 4 were quantified using capture probe 1 and detection probe 1; RNA transcripts from probes 6 and 8 were quantified using capture probe 2 and detection probe 2. Results are presented in Figures 14 and 15. Figure 14(a) shows the amount of RNA produced using probes 2 and 3 (left hand pair of columns) or probes 2 and 4 (right hand pair of columns), in a transcription-only reaction. Figure 14(b) shows the amount of RNA produced in a reaction requiring extension, followed by transcription,

using probe 3 and RNA1 (left hand pair of columns) or probe 4 and RNA 1 (right hand pair of columns). For each pair of columns, the left hand column shows the yield for the full reaction, and the right hand column shows the yield for the control reaction. In both 14(a) and 14(b) a hex moiety was located downstream of the T7 promoter, as indicated in Figure 13(a).

Figure 15 shows the amount of RNA produced using a template without hex (a) or a template with hex (b). a(i) shows the results using probes 5 and 6, a(ii) shows the results using probes 6 and 7, b(i) shows the results using probes 5 and 8, b(ii) shows the results using probes 7 and 8.

5.6 List of oligonucleotides

Probe 1 Seq ID No. 21

5' TGCCTCCTTGTCTCCGTTCTGGATATCACCCGATGTGTCTCCCTATAGTG ATCGTATTAATTTC 3'

Probe 2 – as Seq ID No. 3, Example 1

Probe 3 – as Seq ID No. 5, Example 1

Probe 4 (as probe 3 above, but with **H** = Hexaethylene glycol)

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCHGTTCTCTCTTCCTATAGTGAG TCGTATTAATTTCTGCCTCCTTGTCTCCGTTCTGGATATCACCCGATGTGTCTCC C 3'

Probe 5 Seq ID No. 22

5' GGGAGAGAGAGCGCTGAGGCTTGAGAGGAGAGACCGGAAGACGA 3'

Probe 6 – (as Seq ID No. 20, but without Hex)

Probe 7 Seq ID No. 23

5' GGGAGAGAGAGCGCTGAGGCT 3'

Probe 8 (H = Hexaethylene glycol) – as Seq ID No. 20

Capture Probe 1 – as Seq ID No. 17, Example 3

Detection Probe 1 – as Seq ID No. 18, Example 3

Capture Probe 2 – as Seq ID No. 6, Example 1

Detection Probe 2 – as Seq ID No. 7, Example 1
5' GGATATCACCCG3' (3' alkaline phosphatase)

Example 6

This example compared the stepped amplification of 50 amol of a DNA primer using two linear DNA templates either containing or not containing chemical linkers.

6.1 Preparation of oligonucleotides

All oligonucleotide probes were prepared and purified as described previously.

6.2 Synthesis of RNA off hybridised oligonucleotide

For reactions using probes with chemical linkers, the following method was used. Hybridisation was achieved in an assay mixture that contained 1 fmol of probe 1 and 50 amol of a DNA primer (probe 2) complementary to the 3' end of the linear template (probe 1); Bst DNA polymerase (New England Biolabs) (3' → 5' exo-, 4 units); 1 µl dNTP mix (Amersham Pharmacia Biotech). The reaction volume was made up to 18 µl with RNase-free distilled water. The mixture was incubated at 37 °C for 1 hour. After this time, 20 fmol probe 3 was added in a volume of 2 µl, thus making the reaction volume 20 µl. For reactions containing probes without chemical linkers, probes 4 and 5 were used. Control reactions contained probes 1 and 3 only or probes 4 and 5 only.

6.3 Capture and detection of synthesised RNA

20 µl of the assay sample (or a dilution thereof) was processed as described in the previous examples. Results are presented in Figure 16. Figure 16 shows RNA yield (in femtomoles) for "stepped" (i.e. non-cycling) amplification reactions in which the template comprised a blocking moiety in accordance with the invention (A), or used conventional template probes without a blocking moiety (B). All reactions used 1 fmol of first template, and either 20 or 40 fmol of second template, as indicated beneath the columns. The numbers above the columns indicate the amplification factor (top number) and signal: noise ratio (bottom number). As with previous figures, in each pair of columns, the left hand column shows the result for the full reaction, the right hand column shows the results for the control reaction. These show that, to improve the process of stepped amplification, chemical linkers are required in the DNA templates. In this work, an Octanediol linker was located between bases –31/-32 in the first DNA template and a Hexaethylene glycol between bases –31/-32 in the second DNA template. The results of the reactions showed that the signal noise and the amount of RNA transcribed were lower when chemical linkers were not included.

6.4 List of oligonucleotides

Probe 1 (O = **Oct**) as Probe 4, Example 4

5' TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCTCGCTTCCTATAGT GAGTCGTATTAATTTCTCGTCTTCCOGGTCTCTCCTCTCAAGCCTCAGCGCTCTC TCTCCC 3'

Probe 2 as Seq ID No. 22, Example 5

Probe 3 (H = **Hex**) Seq ID No. 24

5' TCTGGTGGCTCGGCTCGCGTGCGTGATCGATGGTTGTCTCTTCCTATA GTGAGTCGTATTAATTTCTGCCTGCTTHGTCTGCGTTCTGGATATCACCCG AGTTCTCGCTTCC 3'

Probe 4 – as Seq ID No. 20, but without Hex

Probe 5 – as Seq ID No. 24, but without Hex

Sequence of transcribed RNA Seq ID No. 25

5' GGAAGAGACAACCAUCGAUCACGCACGCGAGCCGAGCCACCAGA 3'

Capture Probe Seq ID No. 26

5' TCGTGTCTGGTGGCTCGGCTCGCGT (5' biotinylated)

Detection probe Seq ID No. 27

5' GCGTGATCGATG 3' (3' alkaline phosphatase labelled)

Claims

1. A probe molecule comprising single stranded nucleic acid; said probe comprising a single stranded sequence complementary to a target nucleic acid sequence; a single strand of an RNA polymerase promoter sequence, and a blocking moiety, there being from 0 to 50 nucleic acid bases between the blocking moiety and the promoter sequence.

2. A probe according to claim 1, comprising the template strand of an RNA polymerase promoter.

3. A probe according to claim 1 or 2, comprising a –5 sequence adjacent to the 3' end of the promoter sequence.

4. A probe according to any one of claims 1, 2 or 3, comprising a +12 sequence adjacent to the 5' end of the promoter.

5. A probe according to any one of the preceding claims, such that when hybridised to the target, the 3' end of the target is extendible by a DNA polymerase.

6. A probe according to any one of the preceding claims, wherein the target complementary portion is located 3' of the promoter sequence.

7. A probe according to any one of the preceding claims, wherein a blocking moiety is located between position –19 and –68.

8. A probe according to any one of the preceding claims, wherein a blocking moiety is located between position –19 and –38.

9. A probe according to any one of the preceding claims, wherein a blocking moiety is located between position –22 and –35.

10. A probe according to any one of the preceding claims, wherein the blocking moiety comprises a C_2-C_{20} alkyl, alkanol or alkylene residue.

11. A probe according to any one of the preceding claims, wherein the probe comprises a C_3-C_{10} alkyl, alkanol or alkylene residue.

12. A probe according to any one of the preceding claims comprising an octanediol, propanediol or hexaethylene glycol residue.

13. A probe according to any one of the preceding claims, comprising PNA and/or LNA.

14. A probe according to any one of the preceding claims, wherein a target complementary portion of the probe comprises PNA and/or LNA.

15. A method of detecting a nucleic acid sequence of interest in a sample, the method comprising the steps of: contacting a nucleic acid probe molecule in accordance with any one of the preceding claims with a nucleic acid target molecule, which target is the sequence of interest or is formed as a result of the presence in the sample of the sequence of interest; causing extension of the 3' end of the target using the probe as a template, thereby creating a functional double stranded RNA polymerase promoter; causing RNA synthesis from the RNA polymerase promoter, to create an RNA molecule; and detecting directly or indirectly the RNA molecule so produced.

16. A method according to claim 15, wherein the RNA molecule is caused to hybridise to a further probe molecule and extended, creating a further RNA polymerase promoter which causes synthesis of a further RNA molecule, thereby amplifying the amount of RNA produced.

17. A method according to claim 16, wherein the further RNA molecule is caused to hybridise to a second further probe molecule and is extended.

18. A method according to claim 16 or 17, wherein the sequence of the further RNA molecule is substantially similar to that of the original target molecule, such that the further RNA molecule is able to hybridise, under the assay conditions employed, to the original nucleic acid probe molecule.

19. A method according to any one of claims 16, 17 or 18, wherein the target sequence comprises DNA or RNA.

20. A method according to any one of claims 16-19, wherein the target sequence is DNA or RNA formed as a result of the presence in the sample of the nucleic acid sequence of interest.

21. A method according to any one of claims 16-20, wherein the RNA molecule is detected directly or indirectly by means of a labelled binding partner.

22. A method according to claim 21, wherein the labelled binding partner comprises an enzyme, a fluorophore, radiolabel or biochemical label.

23. A method according to claim 21 or 22, wherein the labelled binding partner comprises DNA, RNA, LNA, PNA, or any combination thereof.

24. A kit for use in performing the method of any one of claims 16-23, comprising a probe molecule in accordance with any one of claims 1-14, and packaging means.

25. A kit according to claim 24, further comprising one or more of the following: instructions for performing the method of any one of claims 16-23; a buffer; a DNA polymerase; an RNA polymerase; deoxyribonucleotide triphosphates; ribonucleotide triphosphates; and a labelled binding partner.

26. A method of detecting a nucleic acid sequence of interest in a sample, the method comprising the steps of: contacting a nucleic acid probe molecule in accordance with any one of claims 1-14 with a further probe and with a nucleic acid target molecule,

which target is the sequence of interest or is formed as a result of the presence in the sample of the sequence of interest; causing the further probe molecule and the target molecule to hybridise adjacent each other to the probe molecule, thereby creating a functional double stranded RNA polymerase promoter; causing RNA synthesis from the RNA polymerase promoter, to create an RNA molecule; and detecting directly or indirectly the RNA molecule so produced.

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(81) Designated States *(national)*: AU, CA, JP, US.

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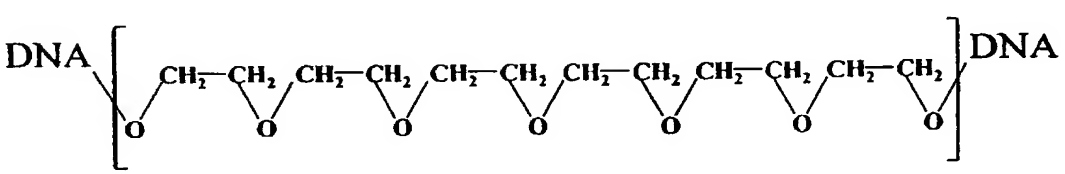
(54) Title: METHOD FOR AMPLIFICATION OF NUCLEIC ACIDS



(57) Abstract: Disclosed is a probe molecule comprising single stranded nucleic acid; said probe comprising a single stranded sequence complementary to a target nucleic acid sequence; a single strand of an RNA polymerase promoter sequence; and a blocking moiety adjacent or substantially adjacent to the promoter sequence, a method of detecting a nucleic acid sequence of interest using the probe; and kits comprising the probe.

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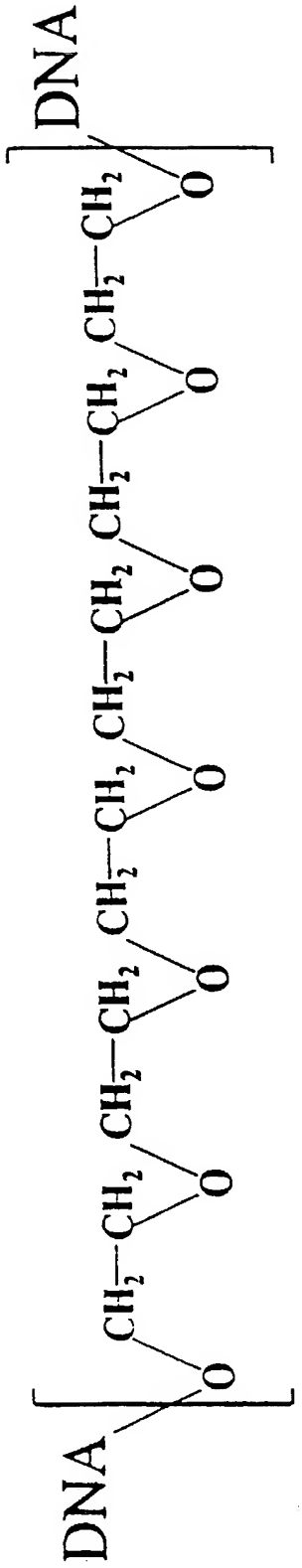
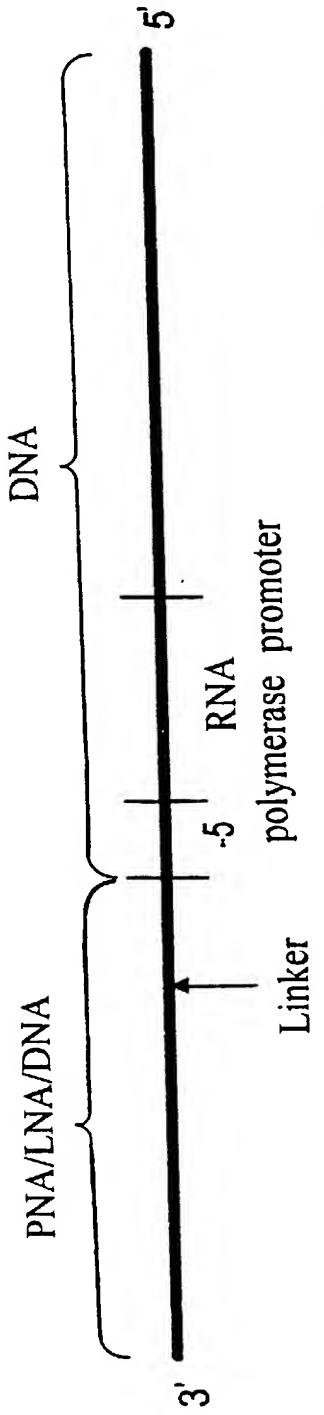


Fig. 1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DNA
RNA
polymerase promoter
-5
Linker
PNA/LNA/DNA
3'

Fig. 2

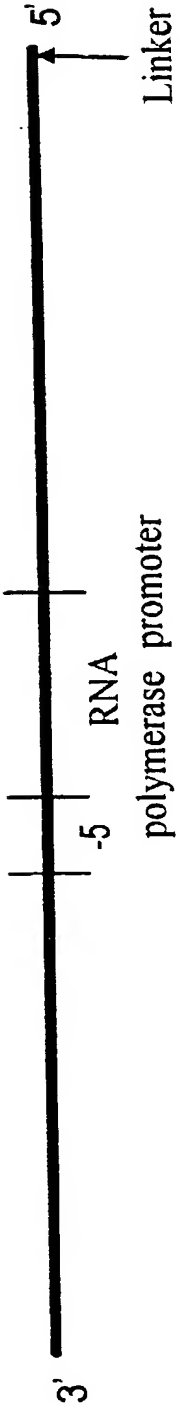
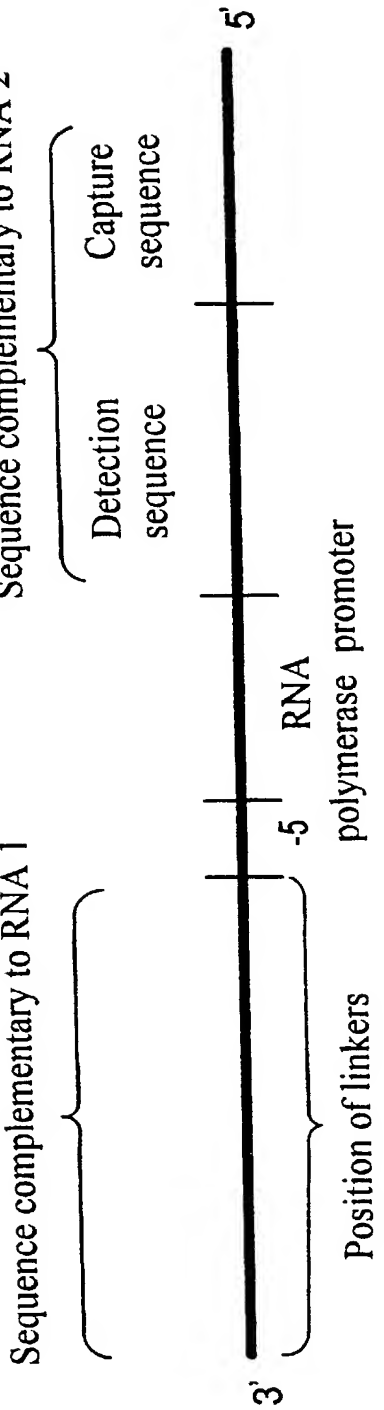

5'
Linker
RNA
polymerase promoter
-5
3'

Fig. 3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Detection sequence
Capture sequence
5'
Sequence complementary to RNA 1
-5
RNA polymerase promoter
Position of linkers
3'

Fig. 4

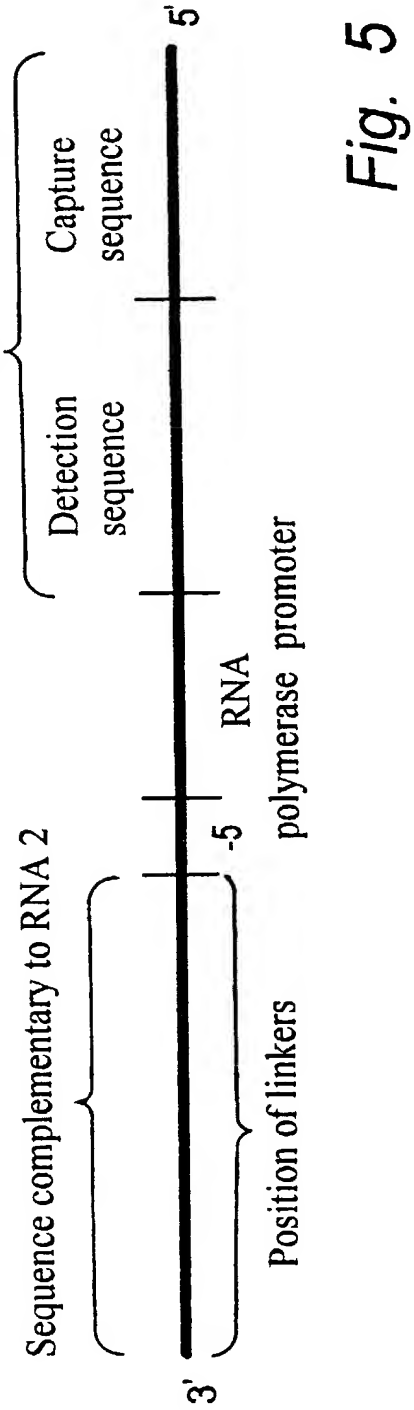

Sequence complementary to RNA 1
Detection sequence
Capture sequence
5'
Sequence complementary to RNA 2
-5
RNA polymerase promoter
Position of linkers
3'

Fig. 5

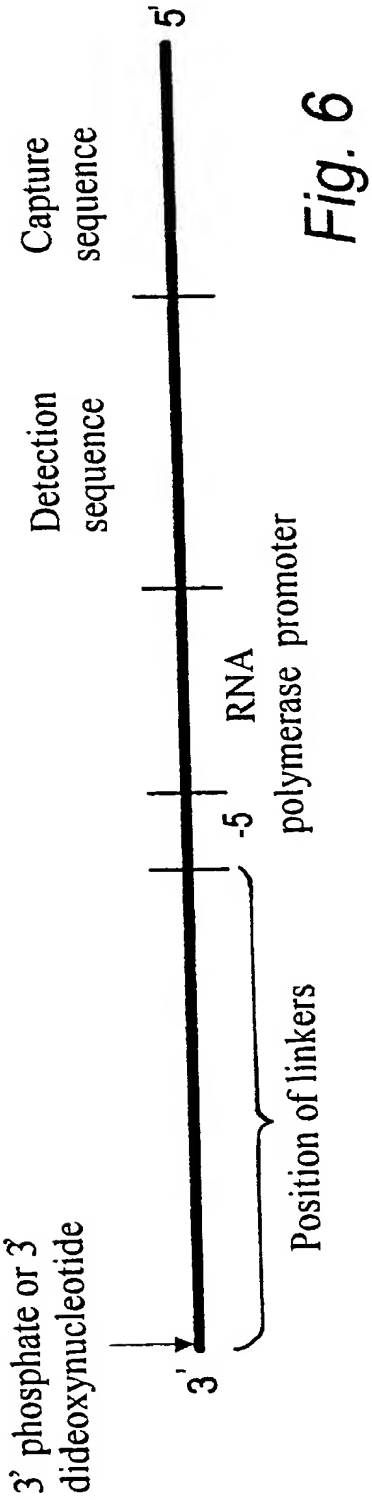

5'
Capture sequence
Detection sequence
RNA polymerase promoter
-5
Position of linkers
3'
3' phosphate or 3' dideoxynucleotide

Fig. 6

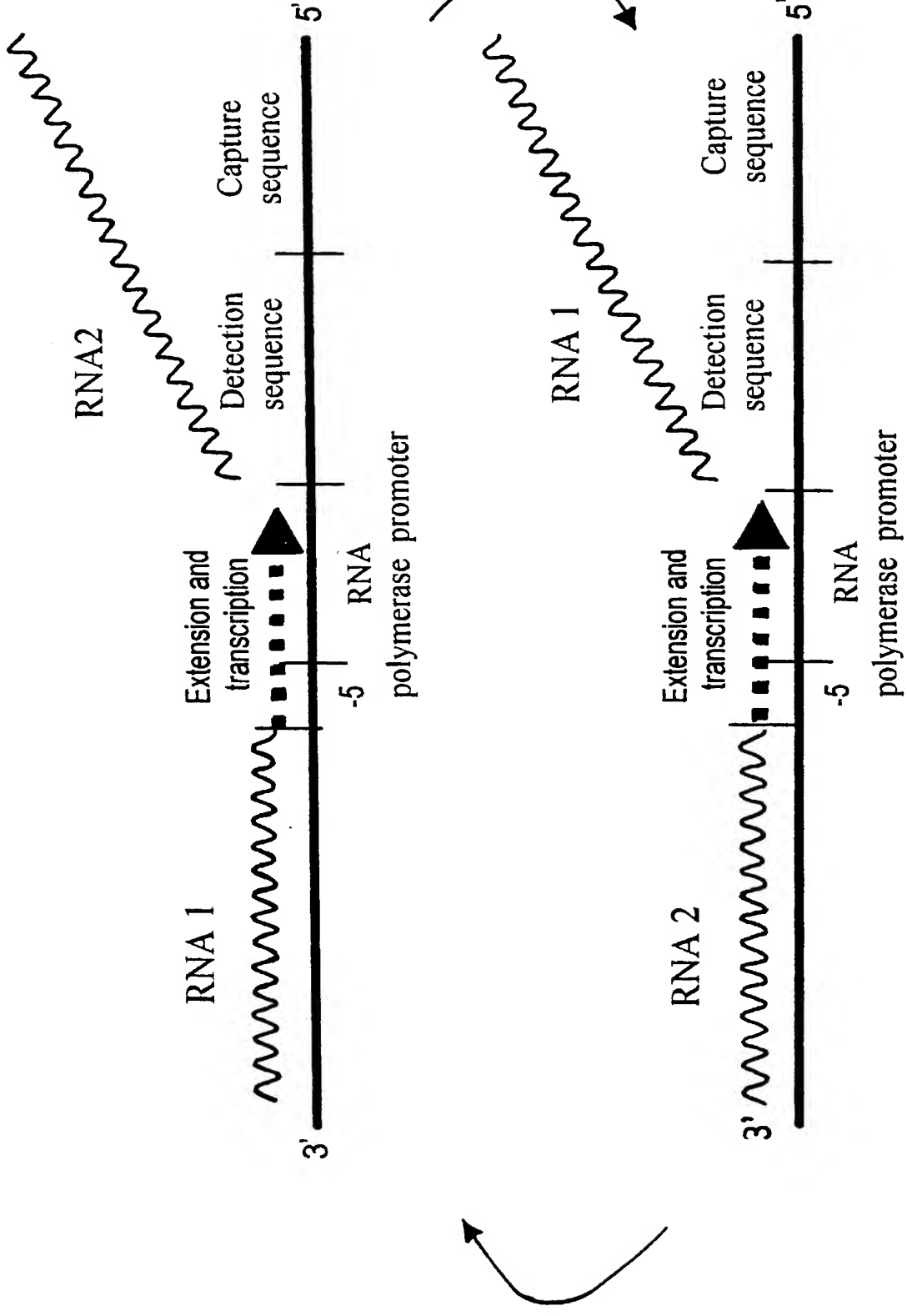
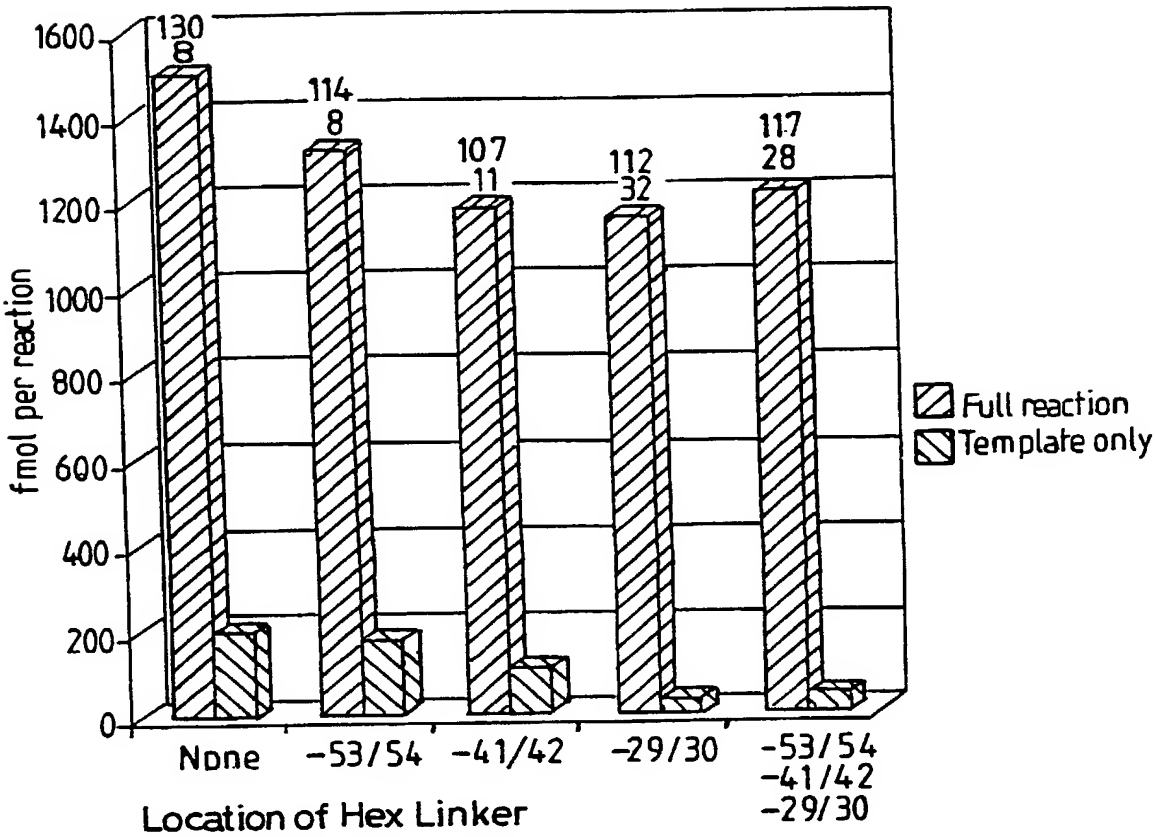

RNA2
Detection sequence
Capture sequence
5'
Extension and transcription
-5
RNA polymerase promoter
RNA 1
3'
RNA 1
Detection sequence
Capture sequence
5'
Extension and transcription
-5
RNA polymerase promoter
RNA 2
3'

Fig. 7


1600
1400
1200
1000
800
600
400
200
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fmol per reaction
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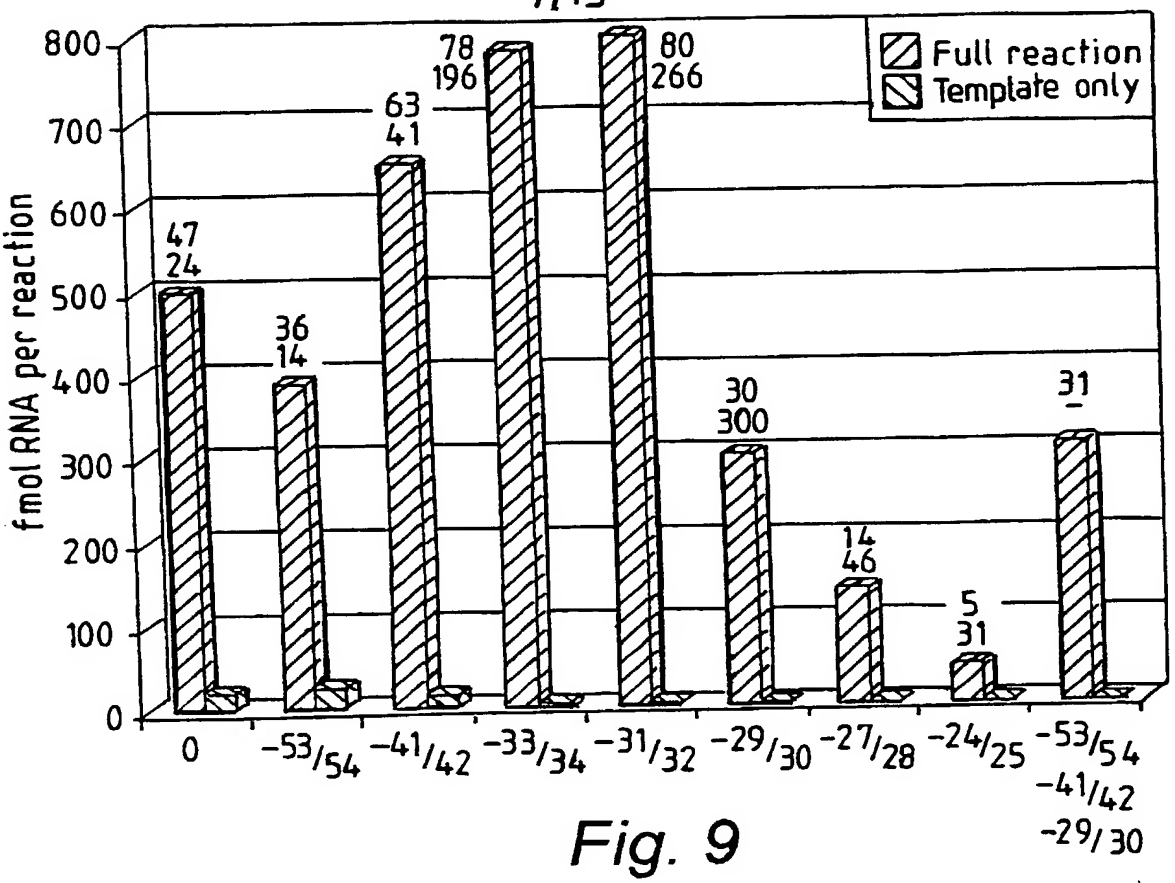
8
114
8
107
11
112
32
117
28
None
-53/54
-41/42
-29/30
-53/54
-41/42
-29/30
Location of Hex Linker
Full reaction
Template only

Fig. 8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700
600
500
400
300
200
100
0
Full reaction
Template only
47
24
36
14
63
41
78
196
80
266
30
300
14
46
5
31
31
0
-53/54
-41/42
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-53/54
-41/42
-29/30

Fig. 9

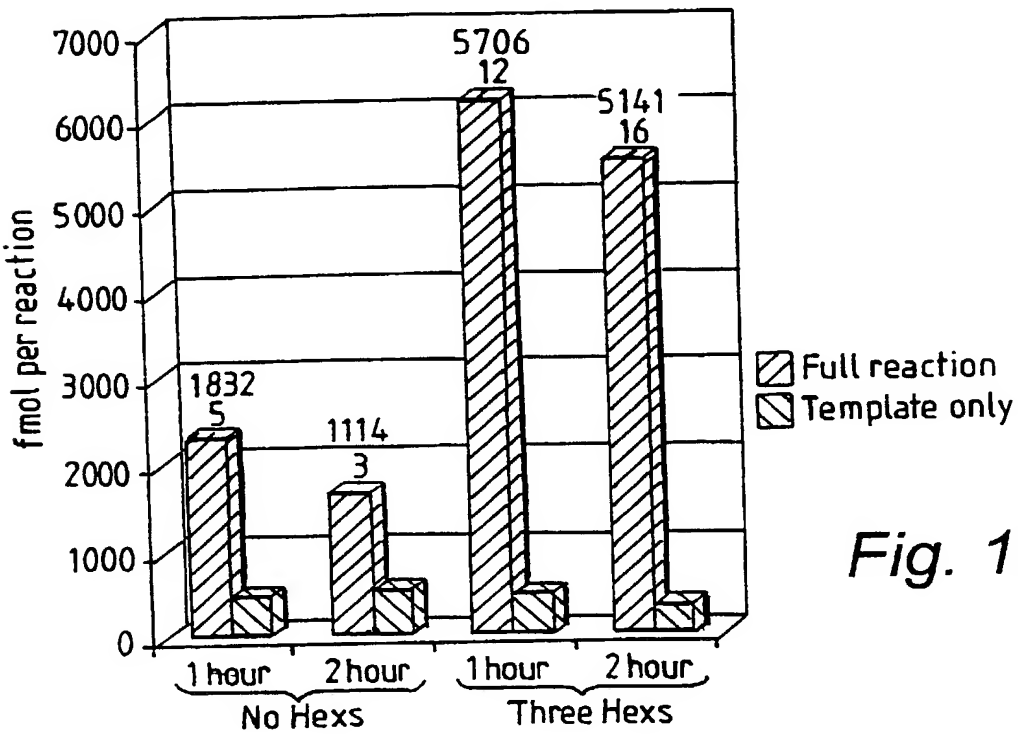

fmol per reaction
7000
6000
5000
4000
3000
2000
1000
0
1832
5
1114
3
5706
12
5141
16
Full reaction
Template only
1 hour
2 hour
1 hour
2 hour
No Hexs
Three Hexs

Fig. 10

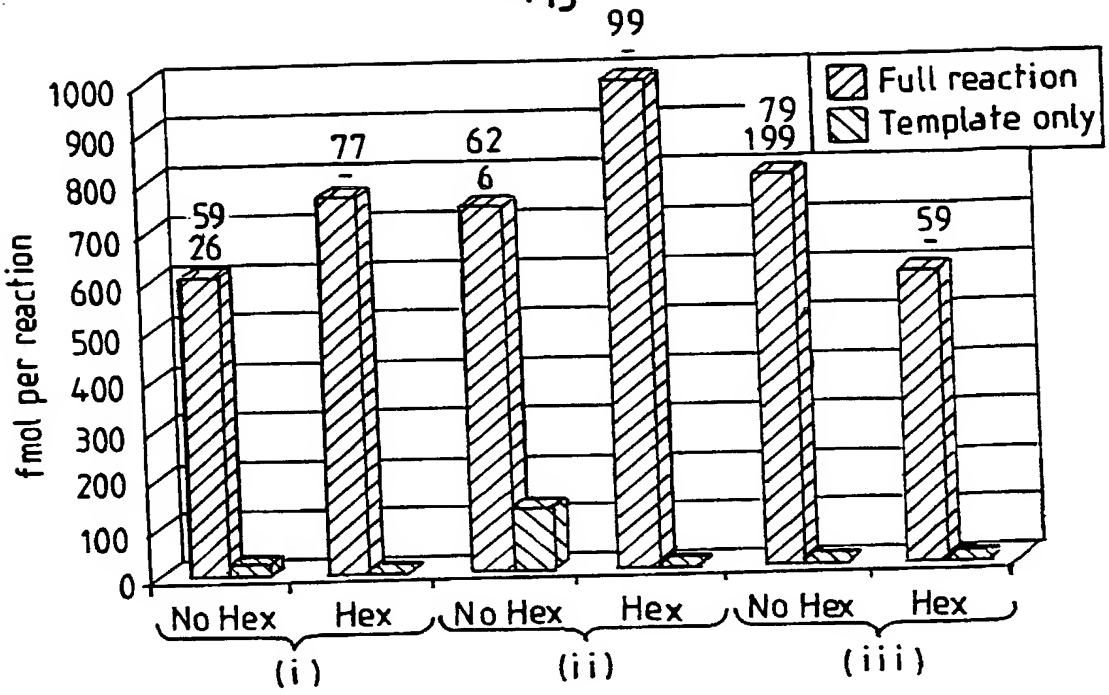


Fig. 11a

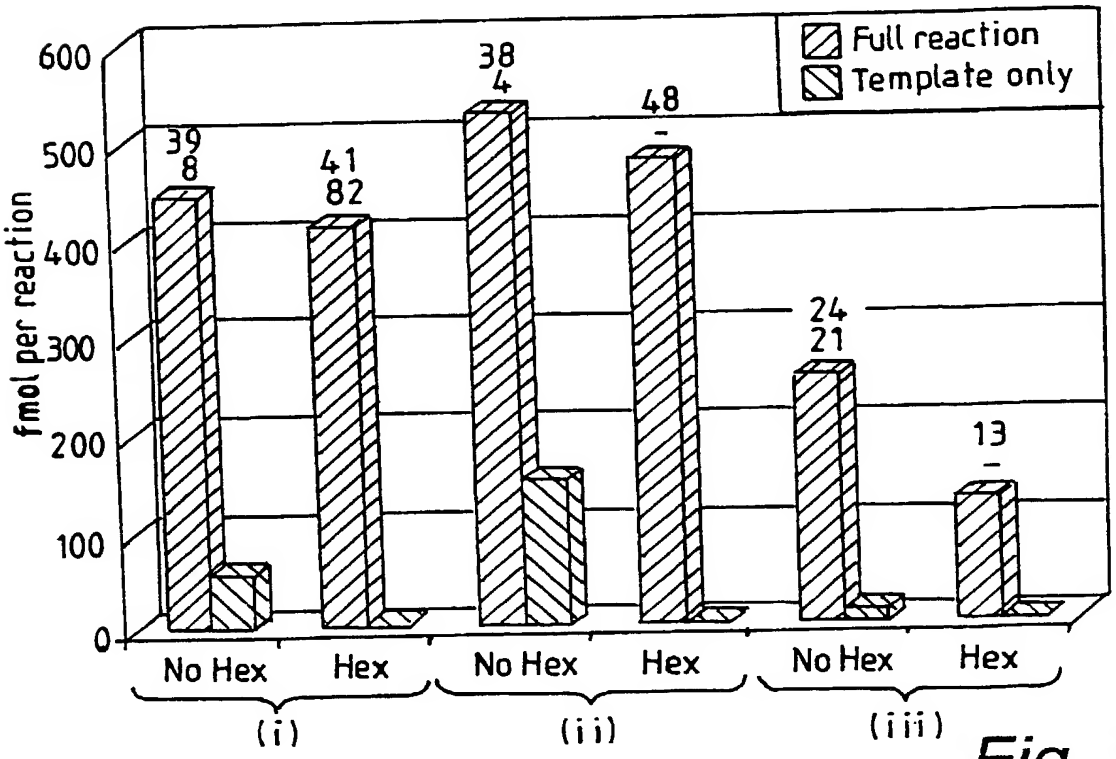


Fig. 11b

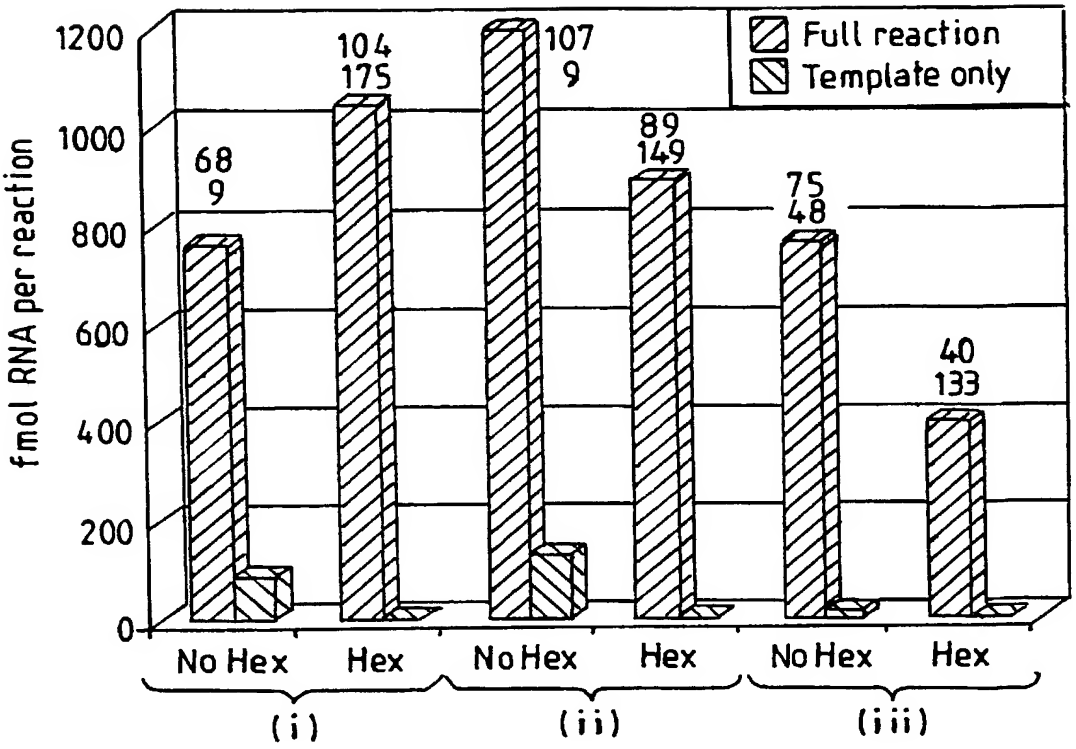


Fig. 11c

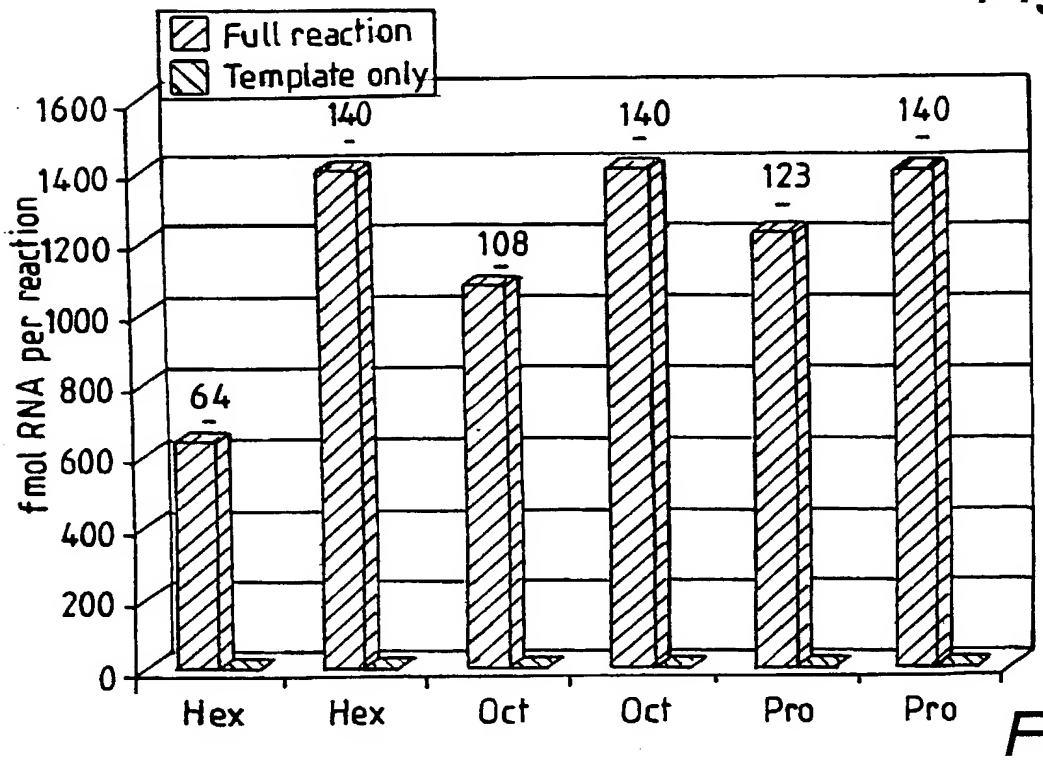


Fig. 12

Hex downstream of T7 promoter

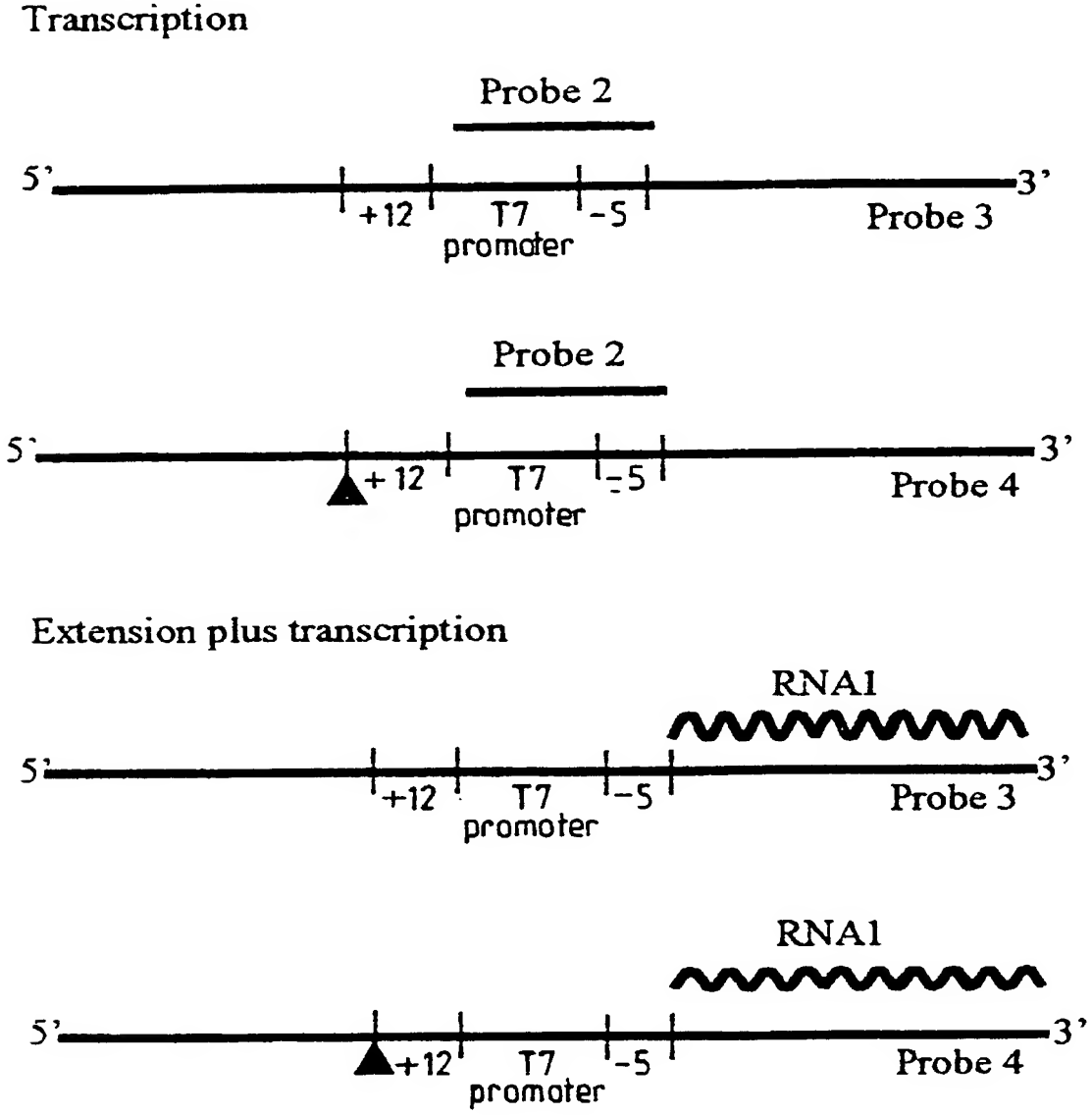
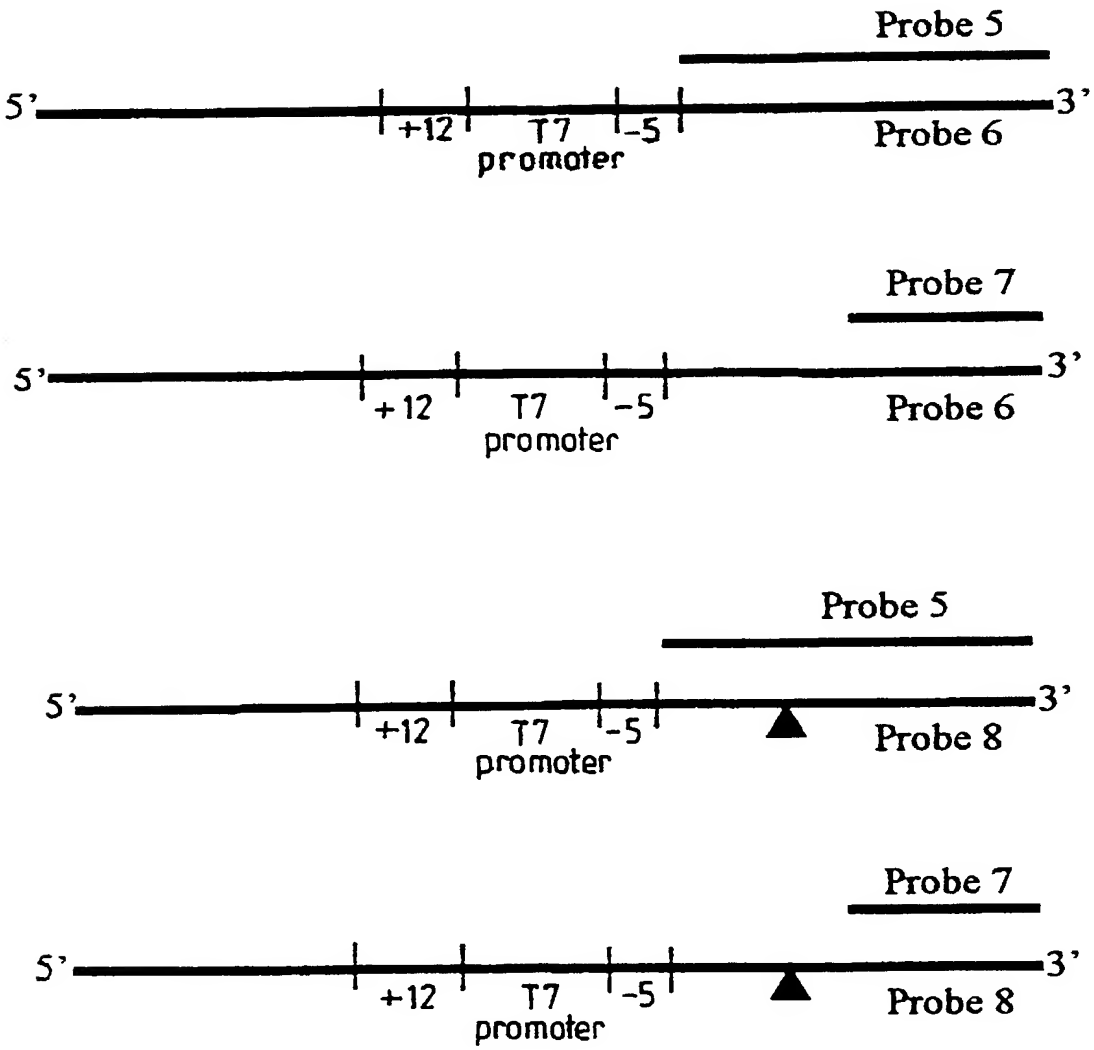


Fig. 13(a)

Hex upstream of T7 promoter

Extension plus transcription



▲ Hex linker

Fig. 13(b)

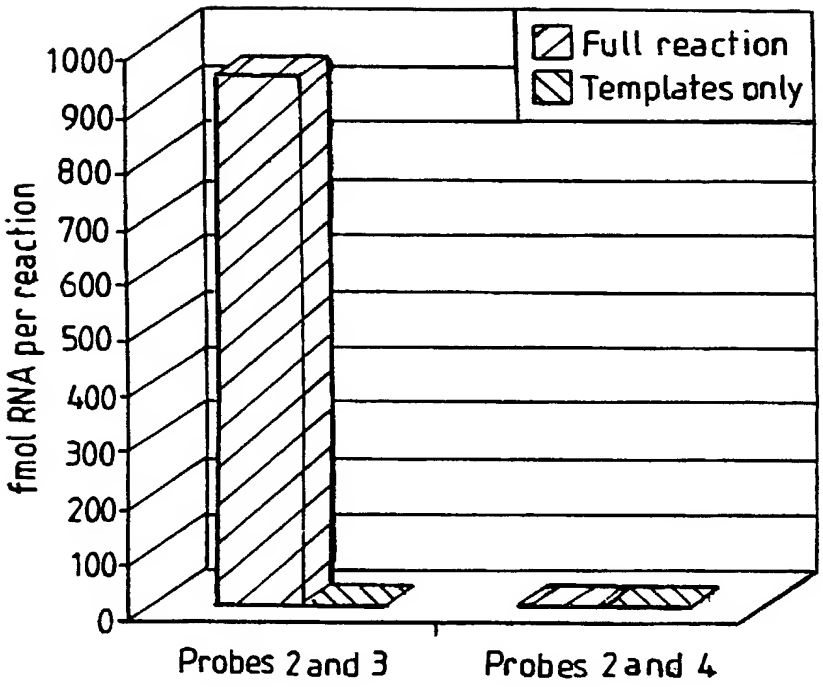


Fig. 14 (a)

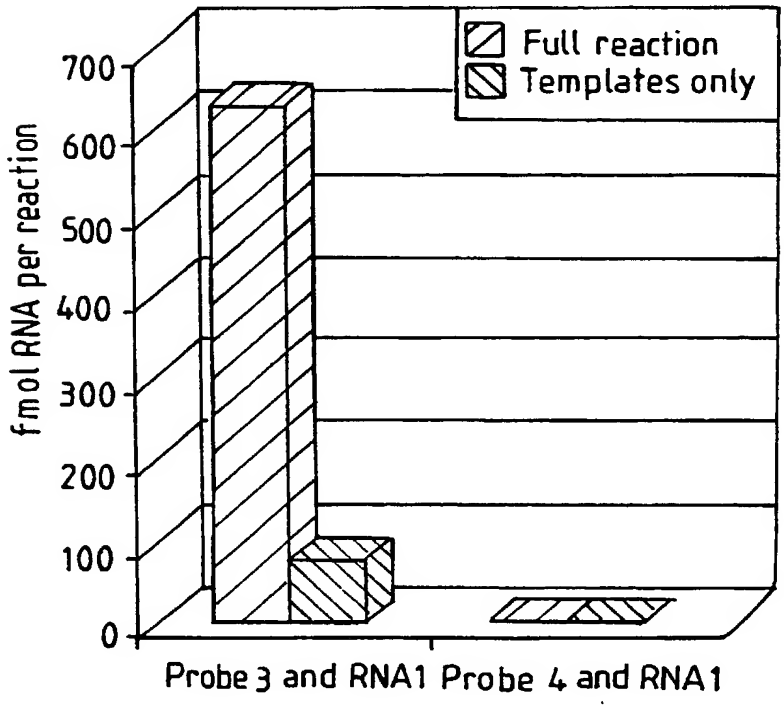


Fig. 14 (b)

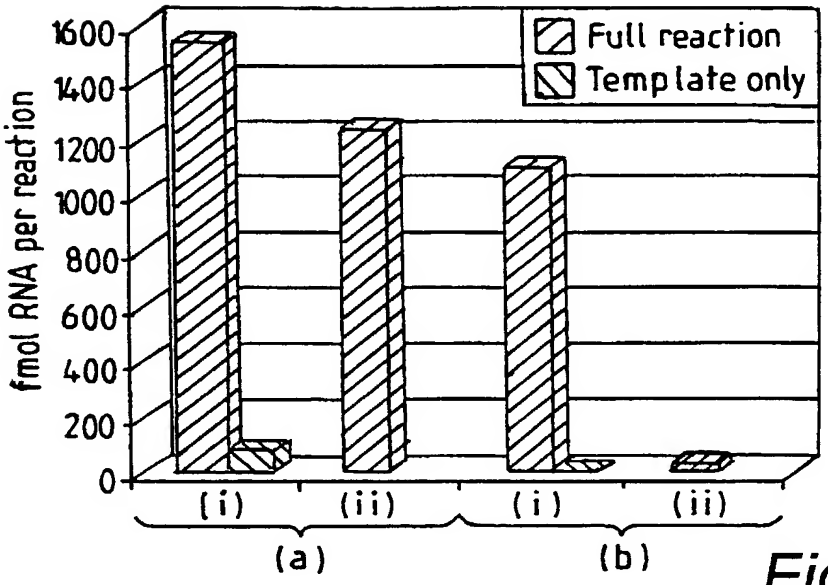
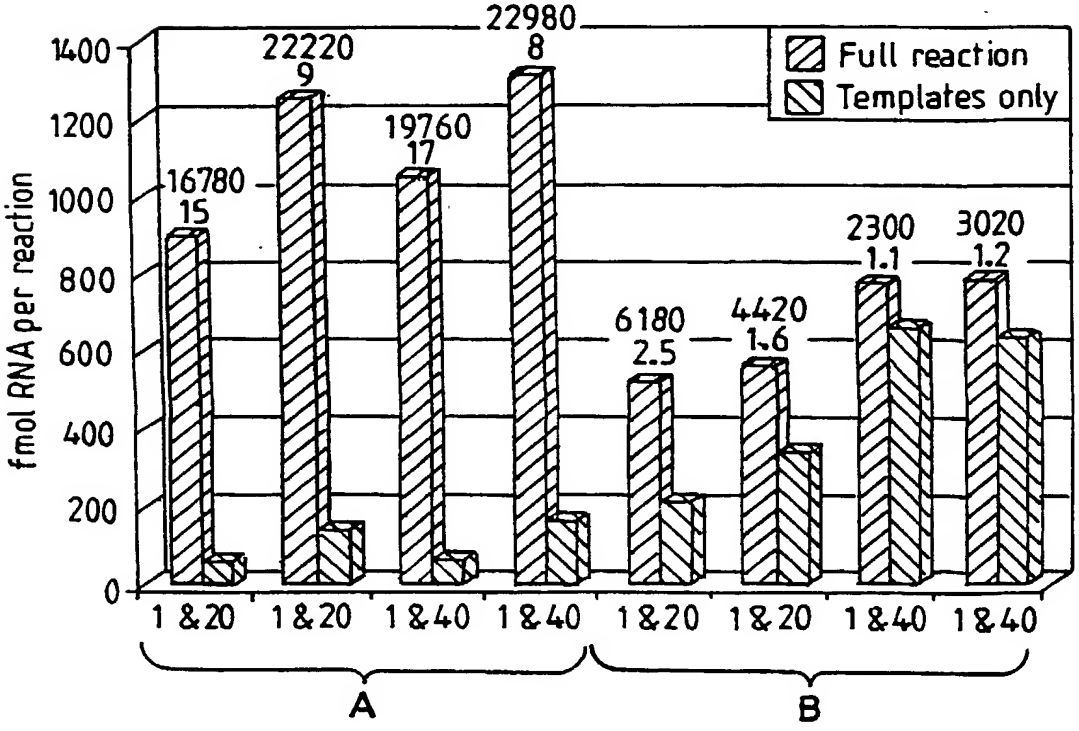

Full reaction
Template only
fmol RNA per reaction
1600
1400
1200
1000
800
600
400
200
0
(i)
(ii)
(i)
(ii)
(a)
(b)

Fig. 15


Full reaction
Templates only
fmol RNA per reaction
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1200
1000
800
600
400
200
0
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15
22220
9
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17
22980
8
6180
2.5
4420
1.6
2300
1.1
3020
1.2
1 &20
1 &20
1 &40
1 &40
1 &20
1 &20
1 &40
1 &40
A
B

Fig. 16

SEQUENCE LISTING

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<120> Improvements in or Relating to Nucleic Acid Amplification

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<141>

<160> 27

<170> PatentIn Ver. 2.1

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atttc 65

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<212> RNA
<213> Artificial Sequence

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<210> 6
<211> 25
<212> DNA
<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic oligonucleotide

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<211> 12
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<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic oligonucleotide

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ggatatcacc cg 12

<210> 8
<211> 67
<212> DNA
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PATENT
ATTORNEY DOCKET NO. 056222-5008-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: **Cytocell Limited *et al.***

Application No.: Not Assigned
(based on PCT/GB00/02946)
Filed: January 29, 2002

Group Art Unit: Not Assigned

Examiner: Not Assigned

For: **Improvements in or Relating to Nucleic Acid Amplification**

BOX SEQUENCE
Commissioner for Patents
Washington, D.C. 20231

STATEMENT ACCOMPANYING SEQUENCE LISTING

Dear Sir:

The undersigned hereby states upon information and belief that the Sequence Listing submitted concurrently herewith does not include matter which goes beyond the content of the application as filed and that the information recorded on the diskette submitted concurrently herewith is identical to the written Sequence Listing submitted herewith.

Respectfully submitted,

MORGAN, LEWIS & BOCKIUS LLP

Dated: January 29, 2002

By: Rachel B. Kapust
Rachel B. Kapust

Customer No. 09629
MORGAN, LEWIS & BOCKIUS LLP
1111 Pennsylvania Ave., NW
Washington, D.C. 20004
Tel: 202-739-3000; Fax: 202-739-3001

SEQUENCE LISTING

<110> Cytocell Limited

<120> Improvements in or Relating to Nucleic Acid Amplification

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<151> 2000-07-31

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COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office

ATTORNEY DOCKET NO.: 056222-5008

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD FOR AMPLIFICATION OF NUCLEIC ACIDS

the specification of which:

is attached hereto; or

was filed as United States application Serial No. _____ on _____ and was amended on _____(if applicable); or

was filed as PCT international application Number PCT/GB00/02946 on 31 July 2000 , as amended under PCT Article 19 on_____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office information which is material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN APPLICATION(S):

COUNTRY (if PCT, indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
Great Britain	9917816.2	29 July 1999	[X] Yes [ ] No
			[ ] Yes [ ] No
			[ ] Yes [ ] No
			[ ] Yes [ ] No

Combined Declaration For Patent Application and Power of Attorney - **(Continued)**
(includes Reference to PCT International Applications)
ATTORNEY DOCKET NO.: 056222-5008

I hereby claim the benefits under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

U.S. PROVISIONAL APPLICATIONS

U.S. PROVISIONAL APPLICATION NO.	U.S. FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or §365(c) of any PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT:

U.S. APPLICATIONS		STATUS (Check One)		
U.S. APPLICATION NO.	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint the registered practitioners of Morgan, Lewis & Bockius LLP included in the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number.

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Combined Declaration For Patent Application and Power of Attorney - **(Continued)**
(includes Reference to PCT International Applications)
ATTORNEY DOCKET NO.: 056222-5008

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1-00

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Listing of Inventors Continued on attached page(s) [X] Yes [ ] No

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4.00

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Listing of Inventors Continued on attached page(s) [ ] Yes [X] No